THE ASPARTIC ACID, GLUTAMIC ACID, PROLINE AND TYROSINE CONTENT OF MEAT [1]

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INTRODUCTION

Previous papers (Schweigert et al., '45; Greenhut et al., '47, '48; Sirny et al., '50) have been concerned with the so-called essential amino acid content of various meat samples. Microbiological methods have been applied successfully in these determinations and have been used extensively by many workers in the analysis of natural materials. The present report on the aspartic acid, glutamic acid, proline and tyrosine content of various cuts of beef, veal, lamb and pork is the first from this laboratory concerned with the amino acids designated as non-essential. Proline was determined with *Leuconostoc mesenteroides* P-60, *Leuconostoc citrovorum* 8081, and *Lactobacillus brevis*, tyrosine with *L. citrovorum*, *L. mesenteroides*, and *Lactobacillus delbrueckii* 3, glutamic acid with *Lactobacillus arabinosus* 17-5, *L. citrovorum* and *L. delbrueckii* 3, and aspartic acid with *L. mesenteroides* only.

EXPERIMENTAL

The meats used for these determinations were lyophilized, ether-extracted preparations made from fresh samples in the summer of 1949. The beef organs were selected on the killing floor of a local packing house to insure that the duplicate samples of each organ came from separate animals.

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The muscle meats were obtained at a local retail store in a chilled state. All samples were stored at —4°C. prior to lyophilization by procedures essentially the same as those described by Sirny et al. ('50). Nitrogen determinations were done by a macro-Kjeldahl procedure.

The medium used for the microbiological determinations was that of Henderson and Snell ('48) with some modifications. Sodium chloride was omitted, and potassium citrate was substituted for sodium citrate equimolarly in order to reduce the sodium content of the medium (Chitre et al., '51). Calcium was used in all media at a level of 20 mg/100 tubes (Meinke and Holland, '50). Reticulogen was added to the medium used for *L. citrovorum* at a level of 0.08 ml/100 tubes, as recommended by Steele et al. ('49). In the assay for glutamic acid, aspartic acid was omitted and was replaced with asparagine at one-half the aspartic acid level (Brickson et al., '48). A pH of 6.8 was used except in the proline assays with *L. mesenteroides,* for which a pH of 6.0 was found appreciably to reduce the lag in the standard curve (Henderson and Snell, '48). The proline assay medium for *L. brevis* was further modified to include L-arabinose and D-glucose at the concentration used by Dunn et al. ('49b) and potassium acetate at 4 gm per 100 tubes in place of the potassium citrate buffer.

The Cannon [2] automatic dispenser and titrator were used for all assays. The assays were conducted with a 2-ml total tube volume and were autoclaved 5 min. at 121°C. The inoculum was from organisms carried on agar and transferred to liquid 24 hours prior to inoculation. Cells were centrifuged and resuspended in 0.9% KCl. Tubes were inoculated aseptically by syringe at one drop per tube and titrated after 65 to 72 hours of incubation at 37°C.

The history of the *Lactobacillus delbrueckii* 3 organism has been previously described by Sirny et al. ('50) and this organism was used in the assay of tyrosine and glutamic

[2] International Instrument Company, Los Angeles, California.

acid. The organism had previously been used in this laboratory for the assay of phenylalanine, isoleucine, leucine, valine, arginine and histidine.

Duplicate hydrolysates of each sample were prepared by autoclaving ½ gm of lyophilized, ether-extracted meat with 20 ml of 3 *N* HCl at 121°C. for 15 hours, a time found to give maximum liberation of the 4 amino acids with each of the assay organisms used (table 1). Recoveries of added amino acids from meat hydrolysates averaged from 96 to 102%.

Because of the work of Chitre et al. ('51) on the inhibition of *L. mesenteroides* and its relationship to the sodium and potassium content of the medium, an acid hydrolysis procedure for tyrosine was used rather than the conventional alkaline hydrolysis. Tyrosine is readily destroyed by acid hydrolysis in the presence of carbohydrate. However, meats appear to be sufficiently low in carbohydrate that this type of destruction does not become a major factor.

Each duplicate hydrolysate was assayed two or more times by each of three organisms for glutamic acid, proline and tyrosine and by one organism for aspartic acid. The samples were run at 6 levels and were read from an average of three standard curves in the rack in which they were incubated.

The standards for proline and tyrosine contained a maximum level of 10 µg per tube, while those of aspartic acid and glutamic acid contained 50 µg per tube, except for 75 µg per tube in the *L. delbrueckii* assay of glutamic acid. The L forms of the amino acids were used in all standards. To eliminate possible salt effects (Camien et al., '47), the salt concentration of the standard was adjusted to make it equivalent to that found in the neutralized meat hydrolysate (Chitre et al., '51).

The standard curves showed no lags except in the case of proline, where *L. mesenteroides* was used as the assay organism. This lag was very slight and encountered in only a few of the assays; however, the values obtained agree closely with those obtained by other organisms (table 2).

TABLE 1

Release of amino acids from beef round with varying time of hydrolysis[1] *and recovery values*
(Value in grams per 100 gm crude protein)

HYDROLYSIS TIME 3 *N* HCl, 15 LB. PRESSURE	GLUTAMIC ACID			ASPARTIC ACID	TYROSINE			PROLINE		
	L.D.3	L.citrov.	L.arab.	L.mes.	L.D.3	L.citrov.	L.mes.	L.citrov.	L.mes.	L.brevis
hours										
1	18.5	11.9	12.1	7.4	3.6	3.1	2.7	3.3	3.7	4.0
2	16.9	14.4	15.0	8.9	3.4	3.3	3.2	3.8	4.2	4.2
3	16.0	15.4	15.7	9.4	3.8	3.5	3.4	3.9	4.1	4.0
10	17.5[2]	16.2	15.2	9.9	3.8	3.6	3.5	4.0	3.9	3.9
15	16.3	15.7	15.4	10.5	3.6	3.6	3.4	3.9	3.9	3.9
25	15.5[2]	16.0	16.4	10.5	3.6	3.6	3.5	3.9	3.9	4.0
Ave. % recovery, 15 hours hydrolysate	102	102	102	101	100	100	100	96	97	99
No. of assays for recovery	7	6	5	6	5	4	5	4	4	4

[1] Values unless otherwise indicated represent one of two or three assays.
[2] One assay only.

TABLE 2

Analysis of meats

(Values in grams per 100 gm of crude protein)

CUT OF MEAT	CRUDE PROTEIN IN MEAT		GLUTAMIC ACID					ASPARTIC ACID		TYROSINE					PROLINE				
	Fresh	Lyophilized	L.D.3	L. citrov.	L. arab.	Ave.	% in fresh meat	L.mes.	% in fresh meat	L. mes.	L. citrov.	L.D.3	Ave.	% in fresh meat	L. citrov.	L. brevis.	L. mes.	Ave.	% in fresh meat
No. of assays			7	6	5			6		5	4	5			4	4	4		
Veal																			
Round	22.5	82.9	15.2	17.1	15.9	16.0	3.6	9.9	2.2	3.5	3.6	3.4	3.5	0.79	4.0	3.6	3.8	3.8	0.85
Shoulder	19.7	85.3	16.5	17.1	16.1	16.6	3.3	9.9	1.9	3.4	3.5	3.4	3.4	0.67	4.3	4.1	4.1	4.2	0.83
Lamb																			
Leg	18.8	82.3	17.2	17.1	16.8	17.1	2.2	10.3	1.9	3.5	3.7	3.6	3.6	0.68	4.2	4.1	4.3	4.2	0.79
Shoulder	17.7	82.7	16.7	17.1	16.3	16.7	3.0	10.2	1.8	3.4	3.6	3.5	3.5	0.62	4.1	4.1	4.2	4.1	0.73
Pork																			
Ham	17.9	85.1	16.2	16.8	16.4	16.4	2.9	9.9	1.8	3.5	3.6	3.5	3.5	0.63	4.2	3.9	4.1	4.1	0.73
Shoulder	16.2	84.8	17.6	17.7	16.7	17.3	2.8	10.1	1.6	3.7	3.7	3.5	3.6	0.58	4.3	3.9	4.1	4.1	0.66
Beef																			
Round	21.6	86.9	15.8	16.9	14.9	15.8	3.5	9.8	2.1	3.3	3.5	3.5	3.4	0.73	3.8	3.5	3.8	3.7	0.80
Shoulder	21.6	84.7	15.4	17.2	16.4	16.3	3.5	9.8	2.1	3.3	3.4	3.5	3.4	0.73	3.9	3.8	3.8	3.8	0.82
Brain	11.4	70.3	14.0	15.4	14.2	14.5	1.7	10.7	1.2	3.9	4.0	4.0	4.0	0.46	4.4	4.6	4.4	4.5	0.51
Brain	10.4	70.3	13.6	14.6	14.4	14.2	1.5	11.0	1.1	3.9	4.0	4.0	4.0	0.42	4.4	4.3	4.4	4.4	0.46
Heart	17.0	78.7	16.2	15.9	15.9	16.0	2.7	10.2	1.7	3.6	3.6	3.6	3.6	0.61	4.6	4.6	4.6	4.6	0.78
Heart	14.9	77.4	16.4	17.3	16.6	16.8	2.5	10.3	1.5	3.5	3.6	3.5	3.5	0.52	4.8	4.8	5.1	4.9	0.73
Kidney	16.7	79.7	12.8	13.2	12.6	12.8	2.1	9.8	1.6	3.6	3.5	3.7	3.6	0.60	5.1	4.9	5.0	5.0	0.83
Kidney	15.4	76.0	13.5	13.9	13.1	13.5	2.1	10.1	1.6	3.6	3.7	4.1	3.8	0.59	5.1	4.9	4.9	5.0	0.77
Liver	18.9	67.7	13.5	14.5	13.7	13.9	2.6	11.2	2.1	3.8	4.1	4.2	4.0	0.76	5.0	5.0	4.9	5.0	0.95
Liver	19.0	73.9	12.9	14.3	12.3	13.2	2.5	10.6	2.0	3.8	3.7	4.1	3.9	0.74	5.3	5.3	5.5	5.4	1.0
Tongue	18.7	83.2	17.5	16.4	16.2	16.7	3.1	10.0	1.9	3.4	3.4	3.4	3.4	0.64	5.2	5.3	5.6	5.4	1.0
Tongue	15.3	82.4	17.2	17.4	16.7	17.1	2.6	9.9	1.5	3.1	3.3	3.4	3.3	0.50	5.5	5.6	5.6	5.6	0.86

Values for aspartic acid in some assays were found to be higher at the lower concentration of the standard curves than at the higher ones.

In the standard curves for all the organisms and amino acids the average difference between the blank and maximum value was approximately 2 to 3 ml of 0.1 *N* NaOH.

The values reported in table 2 are the average values of all assays with a given organism. All values, unless otherwise indicated, are expressed as grams amino acid per 100 gm of crude protein, calculated to 16% nitrogen.

RESULTS AND DISCUSSION

Glutamic acid

The glutamic acid values obtained by the use of *L. delbrueckii* 3 were in good agreement with those obtained with *L. arabinosus* and *L. citrovorum* (table 2). The crude protein of pork, lamb, veal and beef muscle meats contained from 15.8 to 17.3% glutamic acid, with beef tongue and heart within the range of the muscle meats. The beef organs were lower, with the lowest values for kidney, liver and brain being 12.8, 13.9 and 14.2%, respectively.

Hier et al. ('45), using *L. arabinosus* as the assay organism, reported that beef muscle contains 15.2% glutamic acid on an ash, water, fat-free basis, which would be 14.8% of this amino acid on a crude protein basis.

Schweigert et al. ('51), using *L. arabinosus* and *L. mesenteroides,* reported pork shoulder, lamb shoulder and lamb leg to contain 14.7, 15.1, and 13.5% glutamic acid, respectively.

Dunn et al. ('49a), using *L. arabinosus,* reported for raw beef 15.1% and whole raw ham 14.8 and 15.5% glutamic acid; heat-processed beef and ham contained between 13.8 and 14.8% glutamic acid.

Aspartic acid

Aspartic acid was determined with only one organism, *L. mesenteroides.* The average aspartic acid content of the

muscle meats of beef, veal, pork and lamb was 10.0%, with a range of 9.8 to 10.4%, while that of liver was 10.9%. The other organs tested fell within the range of the muscle meats (table 2).

We obtained a value of 9.8% for the aspartic acid content of beef muscle, while Dunn et al. ('49a), also using *L. mesenteroides,* reported a value of 9.2%. Our value of 9.9% for the aspartic acid content of ham also appeared to be in fair agreement with the values of 9.1 and 9.5% reported by Dunn et al. ('49a).

Schweigert et al. ('51), using *L. mesenteroides,* reported an aspartic acid content of 8.34% for pork shoulder, 7.78% for lamb leg and 8.7% for lamb shoulder. Our values for these respective cuts of meat were 10.1, 10.3 and 10.2%.

Tyrosine

The values for the tyrosine content of the various meat samples obtained by the three organisms were in good agreement (table 2). The average tyrosine content of the muscle meats was similar for beef, veal, lamb and pork, being 3.5% with a range of from 3.4 to 3.6%. Beef brain and liver contained 4.0% tyrosine, while the other organs contained amounts similar to those in the muscle meats.

Schweigert et al. ('51), using *L. arabinosus, L. mesenteroides* and *L. citrovorum* as the assay organisms, reported lamb leg and shoulder to contain 3.23 and 3.29% tyrosine, respectively. The same authors reported pork shoulder to contain 3.53% tyrosine.

Gunness et al. ('46), using *L. delbrueckii,* reported the tyrosine content of beef muscle to be 3.4% and that of beef brain to be 3.8%.

Proline

The agreement among the values for the proline content of individual meat samples determined with different organisms was good.

The proline content of the muscle meats ranged from 3.7 to 4.2%, while that of the organs ranged from 4.4 to

5.6%. The proline content of beef tongue, 5.6%, was the highest encountered in these assays.

Schweigert et al. ('51), using *L. mesenteroides* and *L. brevis* as the assay organisms, reported the proline content of pork shoulder, lamb leg, and lamb shoulder to be 4.25, 4.33 and 4.55%, respectively. The values obtained by us for the respective samples were 4.1, 4.2 and 4.1%.

SUMMARY

Leuconostoc mesenteroides P-60, *Leuconostoc citrovorum* 8081, and *Lactobacillus brevis* were used for the determination of proline in meats. *L. citrovorum, L. mesenteroides,* and *Lactobacillus delbrueckii* 3 were used for similar determinations of tyrosine. *Lactobacillus arabinosus* 17-5, *L. citrovorum* and *L. delbrueckii* 3 were used for glutamic acid, while only *L. mesenteroides* was used for the determination of aspartic acid.

The average glutamic acid content of the skeletal muscle tissues was 16.5% of the crude protein, while the average aspartic acid content of the same tissues was 10.0%. The average tyrosine and proline content of the same tissues was 3.5 and 4.0%, respectively.

The average glutamic acid, aspartic acid, tyrosine and proline contents of the various organs were 14.9, 10.4, 3.7 and 5.0%, respectively.

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THE OCCURRENCE OF A CALCINOSIS SYNDROME IN THE COTTON RAT[1]

I. THE EFFECT OF DIET ON THE ASH CONTENT OF THE HEART

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ONE FIGURE

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INTRODUCTION

A colony of cotton rats *(Sigmondon hispidus hispidus)* is maintained in this laboratory for use in a study of the effect of nutrition on the production of dental caries. During the past three years autopsy of the experimental animals has shown that this species, as raised in our colony, is affected by a calcinosis syndrome which is primarily associated with the consumption of partially purified diets. The lesions observed in our cotton rats are similar to those recently described by Harris and Wulzen ('50) in guinea pigs fed diets deficient in the anti-stiffness factor. Since many of the dietary factors which modify the course of the syndrome in guinea pigs behave similarly with cotton rats, it is felt that a report of our studies may help elucidate the cause of the calcinosis syndrome found in the two species.

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Source of the experimental animals

There are only a few laboratories in this country which use cotton rats, and they have not reported the occurrence of similar pathology in their animals. Therefore, a brief consideration of the pertinent facts concerning the management of the stock colony is included in this report. The original diet fed to the stock colony from 1944 to 1949 consisted primarily of dehydrated commercial dog food [2] (table 1). After the supply of this diet was exhausted, several different

TABLE 1

Composition of stock colony diets

DIET 1 — 1944 TO 1949		DIET 2 — 1949 TO 1951 [2]	
	%		%
"Ideal" dog food	71.5	Whole yellow corn	30
Yellow corn	11.0	Wheat germ flakes	10
Casein	7.0	Linseed oil meal	18
Alfalfa leaf meal	7.0	Alfalfa leaf meal	15
Corn oil [1]	3.5	Skim milk powder	15
		Sodium chloride	1
DIET 3 — 1951		Meat scraps	7
Rockland guinea pig pellets		Corn oil	4

[1] Occasionally included 0.6% wheat germ oil.

[2] Diet 2 contains approximately 50% carbohydrate, 24% protein, 7% fat, 1.1% calcium, 0.9% phosphorus.

diets were used, with particular attention being given to the mineral and vitamin content. The composition of the second diet, which was used from approximately 1949 to 1950, is given in table 1. During these trial periods it was noted that guinea pig pellets were quite acceptable to cotton rats, and these have been used since the latter part of 1950. These pellets have maintained an excellent reproduction performance in the colony.

Stock colony animals which ranged in age from 6 months to one and one-half years have been routinely autopsied. Although these animals occasionally showed the presence of

[2] Ideal.

pathological changes in the kidney, liver, and uterine horns, the cardiac and skeletal muscles were normal. These tissues, especially the cardiac muscle, became progressively calcified in our weanling cotton rats which consumed diets of partially purified foods (Constant et al., '52).

EXPERIMENTAL AND RESULTS

Weanling cotton rats were housed in individual wire cages and received food and water ad libitum. They were given two drops of haliver oil weekly.

The basal diet consisted of 24% vitaminized casein,[3] 67% sucrose, 4% salts IV,[4] and 5% corn oil. Modifications of this diet were made by altering the sucrose level. This diet (for purposes of brevity referred to as "high Mg basal") contained 0.56% calcium, 0.48% phosphorus and 0.040% magnesium. Data from representative experiments, which show the varied effect of diet on the development of the calcinosis syndrome, as represented by the ash content of the heart, and on growth, are summarized in table 2 and figure 1.

The basal diet supplemented with 4% 1:20 liver extract provided adequate levels of the nutrients required by cotton rats for growth, as recently reviewed by Schweigert ('48), and resulted in weight gain equivalent to that obtained when the stock diet was fed (McIntire et al., '44; Schweigert, '48). Our animals grew well when fed the basal diet with liver extract (fig. 1F) but in 7 to 10 weeks their hearts invariably showed grossly observable pathological changes and a high ash content of 9 to 19% on a fat-moisture-free basis (table 2). When the calcium level was reduced to 0.3% or 0%, the ash

[3] Provides the following levels of B vitamins/kg of diet: 3.8 mg thiamine hydrochloride, 3.8 mg pyridoxine hydrochloride, 4.5 mg riboflavin, 38 mg nicotinamide, 30 mg calcium pantothenate, 1.5 gm inositol, 1.5 gm choline, 450 mg para-aminobenzoic acid.

[4] Hegsted et al., J. Biol. Chem., *138:* 459, 1941. Salts IV at 4% provides 40 mg % of magnesium, 0.56% calcium, and 0.2% phosphorus. By combining the regular salts IV with Mg-free salts IV, it was possible to feed any level of magnesium desired and maintain the same amounts of the other ions.

Casein, at the 24% level, supplies approximately 0.03% Ca and 0.2% P.

TABLE 2

Effect of diet on the ash content of the heart

RATION FED	WEEKS ON EXPER.	NO. OF ANIMALS	% ASH [1] (range)	Ca, P, and Mg levels in diets		
				Ca	P	Mg
				%	%	%
High mg basal with L.E.[2]	7–10	8	9–19	0.58	0.48	0.04
——— (0.3% Ca)	7–10	4	5–6	0.30	0.48	0.04
——— (0 % Ca)	7–10	9	4–6	0.02	0.48	0.04
——— minus L.E.	3–5	6	6–16	0.58	0.48	0.04
Intermediate mineral basal	5	3	6–7	0.32	0.34	0.02
Low mg basal — sucrose	3–5	8	23–36	0.58	0.48	0.01
——— + 10% casein	3–5	3	8–19	0.58	0.56	0.01
——— + 6% gelatin	3–5	3	20–25	0.58	0.48	0.01
——— (0.3% Ca)	4	3	16–25	0.30	0.41	0.01
Low mg basal — dextrin	5–6	11	8–21	0.58	0.48	0.01
——— + vit. E.	5–6	4	11–14	0.58	0.48	0.01
——— + B vitamins	5–6	4	5–14	0.58	0.48	0.01
Low mineral basal	11	5	3–5	0.03	0.27	0.01
——— + $CaCl_2$	11	5	6–13	0.32	0.27	0.01
——— + $CaCO_3$, 1% NaCl	11	4	7–9	0.32	0.27	0.01
——— + $CaCO_3$	11	5	6–24	0.32	0.27	0.01
Oatmeal diets 1 and 2 [3]	5–6	15	3–6	0.03	0.28	0.07
Oatmeal — milk	11	7	5–6	0.50	0.64	0.07

[1] Moisture and fat-free basis.

[2] Four per cent 1: 20 liver extract.

[3] Includes ash data from animals fed oatmeal diet 2 plus 0.4 or 0.8% calcium as $CaCO_3$ or 0.44% P (3 litter mates/group).

content of the hearts was low, 4 to 6%. The femur ash content of animals fed 0.56%, 0.3% or 0% calcium ranged from 66 to 69%, 64 to 65%, and 57 to 61%, respectively. The minerals mobilized from the bones of the low calcium group were not

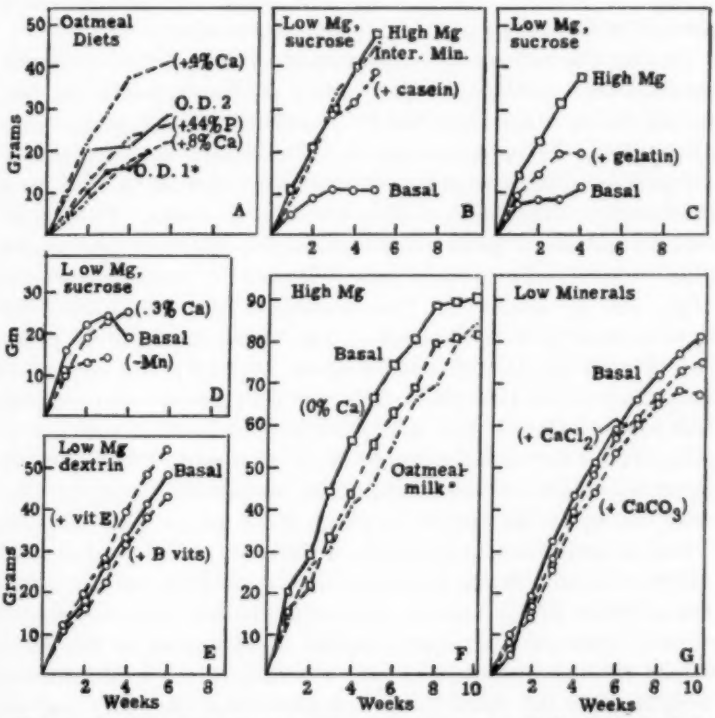

Fig. 1 Growth curves of cotton rats fed oatmeal or partially synthetic diets. (Figs. A–G contain data from representative experiments and, with the exception of the * curves, comparison is made only between litter mates.)

redeposited in the soft tissues. Animals fed the basal diet without liver extract grew well (figs. 1B and 1C) and had 6 to 16% heart ash content in three to 5 weeks. However, reducing the salts IV level from 4% to 2% (intermediate mineral basal — 0.3% Ca, 0.02% Mg) resulted in a low heart ash content of 6 to 7% despite the fact that the magnesium

content of the diet was similarly lowered. These experiments showed that although the basal diet was adequate for the growth of cotton rats, it did not prevent the development of calcinosis. Liver extract at the 4% level did not prevent the lesion. A calcium level of 0.3% and a Ca : Mg ratio of 7.5 : 1 prevented the syndrome for at least 10 weeks.

During the course of these studies we found that partially purified diets with lower (10 mg %) magnesium content (referred to as "low Mg basal — sucrose") consistently produced severely calcified hearts. The heart ash content of animals in several experiments ranged from 23 to 36% on a fat-moisture-free basis within three to 5 weeks. These animals might show good growth gains for the first two weeks, after which their growth was retarded or they lost weight (figs. 1B, 1C and 1D). The mortality rate was high after approximately 4 to 5 weeks. The addition of 10% crude casein resulted in improved growth (fig. 1B) and decreased heart ash (8 to 19% ash). Six per cent gelatin reduced the ash content (20 to 25% ash), but at this level it was not as effective as the slightly higher level of casein. Dietary modifications which imposed additional metabolic demands likewise increased the degree to which the animals were affected. Thus, a concurrent manganese deficiency increased the mortality rate and the calcification of the skeletal muscle, while the addition of 1% sodium acetate to the low magnesium diet greatly increased the pathological involvement of the liver. When the calcium content was reduced to 0.3%, the loss of weight after the third week was prevented (fig. 1D) and the heart ash content was somewhat reduced (16 to 25% ash). However, the animals were in poor health.

Substituting dextrin for the sucrose of this low magnesium basal resulted in nearly normal growth (fig. 1E) and a decreased ash content of the heart (8 to 21% ash). These data also include those obtained from animals fed the dextrin diet supplemented with 0.016 mg % B_{12} or 0.3% cystine. Supplementation of the dextrin diet with 10 mg % alpha-tocopherol did not further decrease the calcinosis (table 2). Supplemen-

tation with twice the level of B vitamins (excluding para-aminobenzoic acid) and including 2 mg folic acid and 0.3 mg biotin per kilogram ration tended to reduce the ash content of the heart (range of 4.8 to 14.4% ash), but the effect was erratic and not completely preventive.

The foregoing experimental diets contained moderate levels of all minerals except magnesium, which was present at a level of 10 mg %. When the level of other minerals was reduced (low mineral basal) by reducing salts IV from 4% to 1% and using Ca-free salts IV, and various calcium salts were added to give 0.3% calcium, striking results were obtained. The animals grew well for 11 weeks (fig. 1G). With the exception of the animals fed the basic calcium salt, the heart ash content was less than 13% at the end of the experimental period.

We found that cotton rats which consume diets with a high content of natural foods have markedly decreased incidence of the calcinosis syndrome. This is shown in part by the stock colony animals, which consumed natural diets with calcium and phosphorus levels of 1.0 to 1.4% and 0.8 to 1.0%, respectively, without manifesting any gross evidence of muscle pathology. The diets containing oatmeal used in these studies showed a marked tendency to protect against calcinosis. Each of the oatmeal diets contained 50% oatmeal. In addition the diets contained: group 1, 8.5% casein and 41.5% dextrin; group 2, 8.5% casein, 14.5% dextrin, 18% sucrose and 9% corn oil; group 2a, the same as group 2 plus 0.44% P as Na_2HPO_4; group 2b, the same as group 2 plus 0.8% calcium; group 2c, the same as group 2 plus 0.4% calcium as calcium carbonate. Animals fed the low calcium diets, groups 1, 2, and 2a, and the high calcium diet, group 2b, grew poorly (fig. 1A). The femur ash contents of these animals were lower than those of group 2c (65%) and were as follows: group 1, 52%; 2, 52%; 2a, 57%; and 2b, 57%. The mobilized minerals were not redeposited in the cardiac muscle, for the highest ash content found was 6% (group 2a). The protective effect of oatmeal was further shown when animals were fed

the oatmeal-milk diet, which consisted of 50% oatmeal and 50% dried whole milk. This diet contained 0.5% calcium and 0.64% phosphorus. At the end of 11 weeks the ash content of the hearts of these animals was still low (5 to 6% ash).

Since decreasing the magnesium content of the diet increased the severity of the calcinosis, and magnesium deficiency in white rats has been shown to produce a several-fold increase in the calcium content of the kidney (Tufts and Greenberg, '37), the kidneys of cotton rats were ashed. The average ash content of the kidneys was similar in all groups, ranging from 5.5 to 7.0%, and was not correlated with the severity of the syndrome.

That nutrition may alter the course of experimentally produced infections has been shown by many studies (Clark et al., '49), although the dietary influence in many cases has not been well defined. In the present experiments cultures of cecal contents for *Salmonella* were negative, as were tests for *Bartonella*. No correlation could be found between the severity of the syndrome and the blood cell picture, including hemoglobin, total red blood and white blood cells, and differential counts. No abscesses have been found in our cotton rats, in contrast to the high incidence found in guinea pigs (Harris and Wulzen, '50). Only rarely did even the cotton rats most severely affected show evidence of pneumonia. Subcutaneous injections of magnesium- or manganese and magnesium-deficient mice with 1:20 homogenates of affected liver or muscle were without effect. Mice fed a magnesium-deficient diet containing 1 to 2% dried feces from affected cotton rats did not develop the syndrome.

Attempts to produce calcinosis in other species have been essentially negative. Diets which produce the severest lesions and maximum stress in cotton rats were without effect when fed to white rats or hamsters for 10 to 12 weeks, and resulted in the sporadic and unreproducible occurrence of cardiac lesions in mice.

DISCUSSION

Petering et al. ('48) noted that oatmeal delayed or alleviated the wrist stiffness syndrome in guinea pigs; Hogan et al. ('50) found that an increased dietary level of phosphate resulted in a more extensive calcification of the soft tissues of their deficient guinea pigs; Van Wagtendonk and Wulzen ('46) were not able to cure or prevent the wrist stiffness syndrome in guinea pigs with vitamin E. Similar observations have been made with regard to the effect of diet on the calcinosis syndrome in cotton rats. Cotton rats, however, do not show evidence of any wrist stiffness. It has been shown further, by the use of cotton rats from our colony, that the ratio and level of calcium, phosphorus and magnesium are important factors in determining the acuteness of the disease. The high level of calcium and phosphorus consumed by stock colony animals and the failure of high levels of magnesium in partially purified diets to prevent the syndrome indicate that natural rations contain a factor or produce digestive conditions which are lacking in the experimental diets. The factor or conditions provided by dextrin or additional casein in alleviating the syndrome cannot be explained at the present time.

SUMMARY

Survey data have been presented on the effect of diet on the development of a calcinosis syndrome which produced a markedly increased ash content of the hearts of cotton rats. The alleviating effect of oatmeal, the detrimental influence of high phosphorus, and the failure of vitamin E to cure the syndrome in guinea pigs apparently apply also to cotton rats. The syndrome in cotton rats was primarily associated with the feeding of partially purified diets. Preliminary studies have failed to establish an infective agent as the cause of this syndrome.

Dietary conditions which increased the severity of the syndrome were (a) increased calcium and phosphorus; or (b) decreased magnesium levels; or (c) basic mineral mixture; or

(d) increased metabolic demands imposed by manganese deficiency or the feeding of sodium acetate. Dietary modifications found to alleviate the syndrome were dextrin or additional protein. Supplementation of partially purified diets with B vitamins, cystine, B_{12} or 1:20 liver extract had little effect on the syndrome. A high level of magnesium did not prevent it.

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THE OCCURRENCE OF A CALCINOSIS SYNDROME IN THE COTTON RAT [1]

II. PATHOLOGY

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ELEVEN FIGURES

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The occurrence of diffuse calcification in cotton rats which consumed partially purified diets has been reported (Constant and Phillips, '52). Although the acuteness and extent of the disease could be modified by altering several dietary factors, the lesions remained essentially the same. Certain similarities were observed between the effect of diet upon the development of the calcinosis in guinea pigs deficient in the anti-stiffness factor and its effect upon the syndrome in our cotton rats. On the basis of the description by Harris and Wulzen ('50), the microscopic appearance of the lesions was similar. The purpose of the present report is to describe the pathologic alterations that have been observed in the tissues of cotton rats affected by the calcinosis syndrome.

EXPERIMENTAL

Material

The experimental procedure and the dietary modifications that alter the course of this syndrome in cotton rats have

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been described (Constant and Phillips, '52). The tissues were fixed in Bouin's solution and stained by either Delafield's hematoxylin and eosin, Lillie's leucofuchsin for Schiff positive material, or Mallory's connective tissue stain.

OBSERVATIONS

Cardiac and skeletal muscle. The heart was the first organ to be progressively calcified. The initial lesion occurred as small foci at the apex of the heart. The lesions appeared grossly as white, linear streaks that coursed parallel to the muscle bundles without involving the coronary arteries. As the lesions progressed, the number of linear streaks increased and extended to the base of the heart (fig. 1). The calcific deposits extended through the entire thickness of the ventricular walls and also involved the auricles. If the involvement was chronic and severe, these white strands, which tended to remain discrete, were located in the adventitia about the great vessels leading from the base of the heart; but arteries outside the thoracic cavity were relatively uninvolved, except infrequently by extension of severe lesions in muscles. The endothelium of the heart remained smooth even in cases where very little normal cardiac muscle remained. In severe cases cutting of the heart was associated with a gritty sensation in the fingers.

White streaks occurred in the skeletal muscle of those animals in which approximately 20% or more of the heart surface was involved. The abdominal, psoas major, caudal flexors and femoral muscles were principally affected in approximately that order. The diaphragm frequently showed similar white streaks, which gave it the appearance of an opened fan. The fascia over the muscles of the inguinal and femoral region was usually calcified to some extent. The psoas muscles frequently contained so many calcified fibers that they appeared as a semi-solid calcified mass. One or two round, white areas 2 to 3 mm in diameter were usually present in the tongue in severe cases.

Microscopically the lesions in the striated muscle developed in stages, as is illustrated in figure 5. The first stage appeared to be a degenerative change which occurred as a swelling and loss of striations of muscle fibers which might be followed by atrophy. The degeneration progressed to necrosis either by liquefaction or fragmentation. While no special muscle bundles were preferentially affected, the smaller skeletal muscle bundles and the outer cardiac muscle bundles of the left ventricle appeared to be more susceptible. The necrosis of the larger muscle bundles tended to start at the periphery. Following necrosis there occurred a proliferation of sarcolemma nuclei and fibrocytes, and an accumulation of small and large mononuclear cells which frequently coalesced to form multinucleated giant cells. There was no pigment accumulation suggestive of vitamin E deficiency according to the description of Filer et al. ('46) nor was there perivascular accumulation of monocytes indicative of a magnesium deficiency (Lowenhaupt et al., '50). There rarely occurred an inflammatory reaction to this necrosis. The lesions were apparently not associated with nerves or blood vessels. The final stage was characterized by the deposition of calcium. By using Lillie's leucofuchsin method, a Schiff-positive material was demonstrated in the affected muscle bundles at the site of the deposition of calcium (figs. 3 and 4). In severely affected hearts there was an increase in Schiff-positive fibrous tissue (fig. 4). Although the entire ventricular wall might be affected, the coronary arteries were uninvolved. The endocardium was generally not affected unless by extension of the lesion from the adjacent muscle (fig. 4).

Liver and gall bladder. The nature of involvement of the liver was unique. The lesions occurred as small (approximately 1 mm) discrete white spots distributed along the edge and surface of the lower half of the left lateral lobe. When the organ was extensively involved, the liver lesions occurred in other lobes. The next susceptible areas were the gall

bladder bed and the site of attachment of the left lateral ligament to the liver.

The majority of animals fed partially purified diets containing sucrose and having a Ca : Mg ratio of approximately 14 : 1 or higher showed some degree of liver involvement. When the Ca : Mg ratio was 56 : 1 and the calcium level was 0.56%, the incidence was approximately 100%. The most extensive liver pathology occurred when this low magnesium diet was supplemented with 1% sodium acetate. The incidence was greatly reduced when dextrin was substituted for sucrose, the Ca : Mg ratio was less than 7 : 1, or the diet contained 50% oatmeal.

A high incidence of cytoplasmic swelling was observed in the hepatic cells of cotton rats fed partially purified diets. A mild to moderate vacuolation of liver cells, as well as evidence of regeneration, was observed, depending upon the severity of the disease. This vacuolation resembled that described by Gillman and Gillman ('49) and was found to be associated with anoxia. Fat vacuoles were seen only occasionally in small numbers. The lesions in the liver apparently progressed through stages of degeneration, necrosis, mononuclear and giant cell reaction and calcification (figs. 8 and 9), as observed in other tissues.

In a few instances only, where the gall bladder was severely involved, were there significant changes in the gall bladder that could be related to the calcinosis. Such alterations were characterized by an empty and shriveled gall bladder with thickened walls. However, of considerable interest was the observation that all animals fed the low magnesium diet supplemented with 1% sodium acetate had distended gall bladders filled with a semi-solid white substance. The gall bladders of approximately 30% of the animals fed a low calcium-high magnesium diet (Ca : Mg ratio of less than 7 : 1) were similarly distended.

Adipose tissue. Grossly observable calcification of fat was caused by only two diets. These were the high magnesium basal (Constant and Phillips, '52) and the low magnesium

basal supplemented with cortisone (unpublished data). The lesions in the mesenteric fat appeared grossly as small (1 mm) discrete white nodules. The retroperitoneal fat frequently contained a large mass of the white substance which cut with a gritty sensation in the fingers. Animals which were fed the cortisone-supplemented diet had masses of calcified subcutaneous tissue over the thoracic wall and neck region. The microscopic appearance of the lesions in adipose tissue (figs. 10 and 11) was similar to that of lesions in other tissues.

Kidney. A few petechiae or minute white spots were occasionally observed, which microscopically proved to be congested glomeruli or dilated tubules. These changes occurred irrespective of the diet or the severity of the syndrome. Rarely, a subserosal lesion resembling those found in other tissues was observed in a severely affected animal. We have not observed basophilic casts in the tubules, as noted in magnesium deficiency in the white rat (Lowenhaupt et al., '50) and in the deficient guinea pigs studied by Harris and Wulzen ('50).

Spleen. There was no correlation between spleen size, the type of diet fed, and the severity of the syndrome (Constant, '51). An increase in megakaryocytes was noted when the diets contained calcium, but their number bore no relation to the severity of the calcinosis. The feeding of partially purified diets usually resulted in an increased deposition of hemosiderin-like pigment.

Pancreas. The microscopic appearance of the pancreas of cotton rats fed a low calcium-oatmeal diet or a partially purified diet with a Ca : Mg ratio of 7 : 1 or less resembled that associated with uremia by Baggenstoss ('48) — dilatation of acini, flattening of epithelial cells and inspissation of the secretion. However, blood urea levels were not abnormal. The pancreas of animals fed diets in which the Ca : Mg ratio was greater than 7 : 1 showed no histopathological changes.

Other organs. In severe cases the urinary bladder frequently showed the typical white streaks of calcification. The

intestine was rarely involved. The cecum was the most susceptible portion of the digestive tract. The thyroid showed no histopathological changes. Although the histologic appearance of cells of the adrenal cortex and medulla was modified slightly, depending on the presence or absence of calcium in the diet, no histopathological changes were observed which could be correlated with the severity of the lesions in other structures. Infrequently, the adrenal cortex of animals which had severe lesions showed the presence of a solitary, deeply basophilic mass. Males which were fed the low magnesium diets and showed poor growth or weight loss frequently had small seminal vesicles, which in one or two animals were so rudimentary as to be scarcely visible grossly. The testes were usually of normal size and color, but occasionally showed mild degenerative changes. In view of the fact that degenerative changes in the gonads were observed only in acutely ill, poorly growing cotton rats, and in view of the observations by Mason ('33) that degenerative changes of the male gonads occur with inanition, it is felt that these changes in the cotton rats were secondary to the development of the syndrome. The lungs of even the most severely affected animals rarely showed any evidence of pneumonia. Abscesses or foci of injection in the muscles or organs have not been a complicating factor in the calcinosis syndrome in cotton rats.

DISCUSSION

Comparison of the pathological changes in cotton rats with those in guinea pigs (Harris and Wulzen, '50) showed a marked similarity of the calcinosis in both species. Cardiac and skeletal muscle cells were the most susceptible in both. The basic lesion was necrosis, with fibrocytic or mononuclear cell reaction or both, giant cells and calcification. Vascular changes were not found to be the cause of the necrosis. There was a relative lack of leukocytic reaction to the myocardial necrosis in both species. The gross and microscopic appearances of the lesions in the liver are similar in both. The atrophy and degeneration of male glands, noted in both

species, was felt in cotton rats to be secondary to development of the calcinosis. The incidence of affectation of endocrine glands was very low in both species.

The main difference between the effect of a diet deficient in the anti-stiffness factor for guinea pigs and that of the partially purified diet fed cotton rats is the considerably greater degree of pathological changes in the kidneys of guinea pigs. Much of the change described as appearing in the kidneys of guinea pigs is suggestive of low magnesium intake, which we have found to be an important factor in the calcinosis syndrome in cotton rats (Constant and Phillips, '52). A high requirement of the guinea pig for magnesium has been shown by Roine et al. ('49). Increasing the magnesium content of the diet permitted normal growth and decreased but did not prevent the calcinosis in cotton rats.

SUMMARY

The pathological alterations occurring in cotton rats affected by a calcinosis syndrome have been described. The similarity of these changes to those found in deficient guinea pigs has been discussed. The syndrome in cotton rats was manifested primarily by necrosis and calcification of the myocardium and skeletal muscle. Other organs may be similarly affected but the incidence and severity with regard to them were considerably less.

Although the severity and acuteness of the disease may be markedly increased by lowering the magnesium content of the diet, no microscopic evidence of magnesium deficiency was found. Histologic examination of various organs supported the results of previous studies in ruling out vitamin E deficiency as the cause of the calcinosis.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Heart which was evaluated 3 + on gross observation. Shows the linear, white streaks. Coronary artery at the right is not affected.

2 Microscopic section of heart in which the lesions are predominantly at the calcification stage. Shows multinuclear cell reaction which was frequently seen in these lesions. Hematoxylin and eosin stain.

3 Microscopic section of heart stained by the leucofuchsin method. Schiff-positive material is concentrated in the area of calcification. Blood vessel walls are not damaged.

4 Severe lesion of left ventricle. Coronary artery is intact. Shows involvement of the endocardium through extension from the cardiac muscle. There is a large increase in fibrous tissue; Schiff-positive material is concentrated at the site of deposition of calcium. Leucofuchsin stain.

5 Microscopic section which shows several stages of the lesion in skeletal muscle: (a) swelling, loss of striations and early liquefaction; (b) fragmentation; (c) early cellular stage; (d) cellular stage with small basophilic granules; (e) large muscle bundles adjacent to affected muscles are for the most part normal. Hematoxylin and eosin stain.

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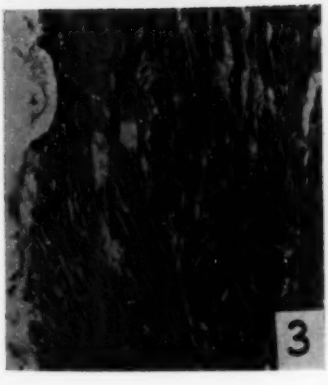
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PLATE 2

EXPLANATION OF FIGURES

6 Microscopic section of femoral muscle showing a predominance of the early necrotic stage of the lesion. Hematoxylin and eosin stain.

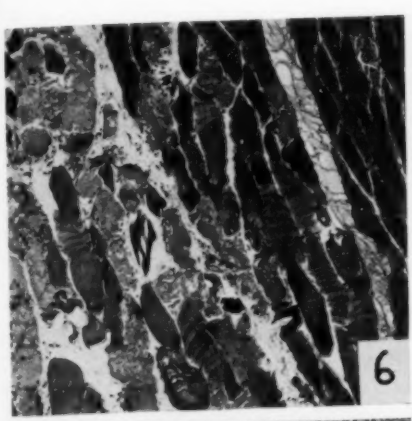
7 Microscopic section of femoral muscle in which the lesions were at a later stage than in figure 1. Cellular reaction and deposition of calcium are similar to those found in cardiac muscle. Shows the tendency of the lesions to remain in a muscle bundle, resulting in linear streaks on gross observation.

8 Microscopic section of liver. Shows focus of necrosis and one medium and one small area of calcification. Figures 8 and 9 show the tendency of the lesions to be located on the periphery of the lobe. Hematoxylin and eosin stain.

9 Shows the cellular stage of a lesion in the liver. This cellular reaction is similar to that found in other tissues. Hematoxylin and eosin stain.

10 Early lesion in adipose tissue. There is a marked fibrocytic and mononuclear cell reaction to the degeneration and necrosis. This section shows several of the multinucleated cells. Hematoxylin and eosin stain.

11 Late stage of the lesion in adipose tissue showing large deposits of calcium. Hematoxylin and eosin stain.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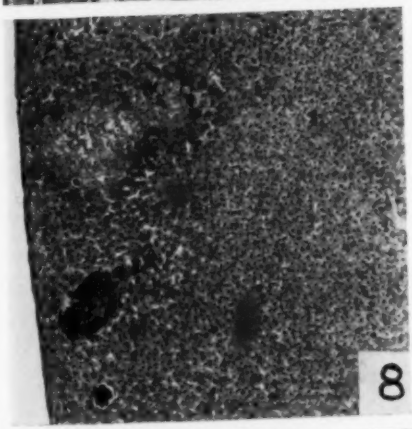
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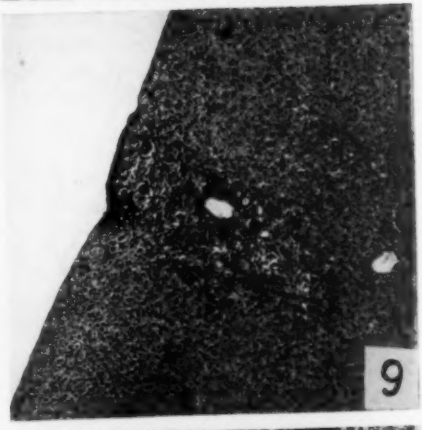
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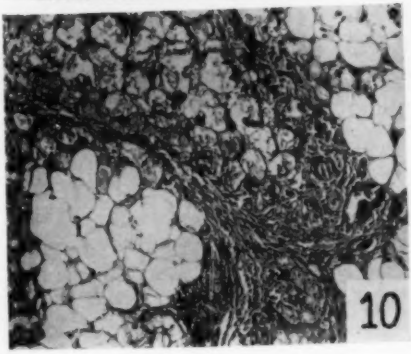
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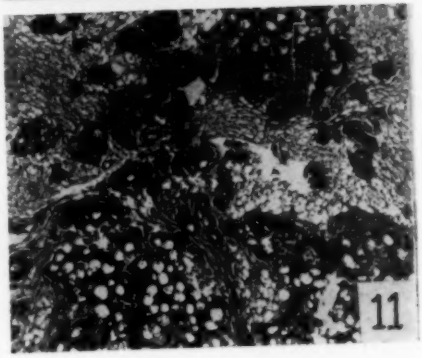
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DAILY DETERMINATION OF PLASMA, SERUM AND WHITE CELL-PLATELET ASCORBIC ACID IN RELATIONSHIP TO THE EXCRETION OF ASCORBIC AND HOMOGENTISIC ACIDS BY ADULTS MAINTAINED ON A CONTROLLED DIET [1]

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ONE FIGURE

(Received for publication March 12, 1952)

Many reports have been made of the ascorbic acid levels of various fractions of blood. Comparison of values reported by different workers is made difficult, not only because of the variety of methods employed, but also because ascorbic acid levels have been analyzed in whole blood, plasma or serum, or white blood cells and platelets. However, no study has been reported in which both serum and plasma ascorbic acid were measured. It has been stated that blood contains no dehydroascorbic acid (Todhunter, McMillan and Ehmke, '50). If this is true, values found for a given blood fraction by a

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method which measures only reduced ascorbic acid should be the same as values found by a method which also measures dehydroascorbic acid. It was the purpose of this study to determine whether serum and plasma ascorbic acid values are the same, and to determine whether total and reduced ascorbic acid values are the same in either serum or plasma.

Although Levine, Dann and Marples ('43) found no evidence of homogentisic acid excretion by infants on vitamin C-free diets, Sealock and Silberstein ('39) reported the following, based on a study of two adults: "On a diet practically free of ascorbic acid the daily ingestion of l-tyrosine resulted in the excretion of significant amounts of homogentisic acid which could be completely prevented by the ingestion of reasonably large doses of crystalline ascorbic acid." While confirming results are lacking, it was felt that it would be desirable to ascertain whether a daily intake of 25 mg of ascorbic acid, said by Göthlin ('34) to be sufficient to prevent scurvy, is also sufficient to maintain normal tyrosine and phenylalanine metabolism, as evidenced by the absence of urinary excretion of homogentisic acid.

DESIGN OF EXPERIMENT

Following the dietary plan of Gifft and Hauck ('46), 4 healthy adult women (one staff member and three graduate students) were placed for 52 days on a basal diet containing 1,000 Cal. per day. In addition, each subject was allowed to take one or more supplemental dietary units containing 500 Cal. but no ascorbic acid. The number of supplemental units was decided upon during the first three days, and kept constant thereafter. Each subject chose two supplemental units, so that the diet for all subjects contained about 2,000 Cal. per day. Representative samples of the food used in the diet were analyzed for ascorbic acid by the method of Loeffler and Ponting ('42) and the basal diet was found to contain 9 mg. Crystalline ascorbic acid was therefore given in addition, so that the total daily intake of ascorbic acid was 25 mg.

The thiamine content of the diet was 600 μg (300 μg in the basal diet and 150 μg per supplemental unit). During the first 31 days of the study, each subject received a daily supplement of 400 μg of crystalline thiamine, making a total daily intake of 1,000 μg of thiamine. The thiamine supplement was discontinued during the remaining 21 days of the experiment. With respect to all other nutrients the diet was adequate.

At the same time that the 4 subjects described above were receiving a controlled daily ascorbic acid intake of 25 mg, a 5th subject (a graduate student who was under a doctor's supervision for a nephritic condition) was receiving a modified diet with a daily intake of about 2,000 Cal., 75 mg of ascorbic acid and 2,600 μg of thiamine. During the first 31 days of the study this subject also received the daily supplement of 400 μg of thiamine.

Blood was collected, after 12 hours of fasting, from a fingertip puncture and analyzed for total ascorbic acid in the serum and plasma by the method of Bessey, Lowry and Brock ('47). This method was also used to determine ascorbic acid in white cells plus platelets and, during the last 7 days of the study, in whole blood. The method of Farmer and Abt ('36) was used to determine reduced ascorbic acid in serum and plasma.

Urine collections were made daily, and each 24-hour collection included the first voiding of the following day. Half of each voiding was treated immediately with one-tenth of its volume of 2% HPO_3 in 2 *N* H_2SO_4. Analyses were made on this treated urine for ascorbic acid by the method of Bessey ('38). Analyses were made on the untreated portion of the urine for homogentisic acid equivalents by the method of Briggs ('22).

A description of the subjects is given in table 1.

RESULTS

Ascorbic acid levels in the blood and urinary excretion of ascorbic acid and homogentisic acid equivalents showed similar changes for each of the 4 subjects receiving 25 mg of

ascorbic acid daily. The complete data from one subject (MLW) are given in figure 1. It can be seen that as the levels of ascorbic acid fall, daily fluctuations become smaller.

The mean values for 10-day intervals for each compound measured are shown for all subjects in table 2. It can be seen that for each subject plasma ascorbic acid levels are lower than serum ascorbic acid levels for both total and reduced ascorbic acid. Furthermore, reduced ascorbic acid levels are lower than total ascorbic acid levels in both serum and plasma. As determined by analysis of variance, the differences are

TABLE 1

Description of subjects

SUBJECT	AGE	HEIGHT	AVERAGE WEIGHT	REMARKS
	yrs.	*in.*	*lb.*	
BLD	28	62¾	108½	Had suffered from poliomyelitis as a child and was lame.
MLW	34	65½	123¾	Chinese.
HHY	36	64	126½	Chinese.
CAS	42	68½	147	
BWC	31	62	113½	Under doctor's supervision for nephritic condition.

statistically significant in each of these comparisons for each individual.

It can also be seen that with a daily intake of 25 mg of ascorbic acid the serum and plasma levels of ascorbic acid were still falling at the end of the experimental period of 52 days. No signs of scurvy were apparent in any of the subjects during this period.

White cell-platelet ascorbic acid levels also declined during the 52-day period, to about half or less of the initial value of those individuals receiving 25 mg of ascorbic acid daily. Conversely, after the first few days there was little change in the excretion of ascorbic acid in the urine. The urinary excretion of homogentisic acid equivalents was not increased

in any subject. In fact, in three of the 4 subjects receiving 25 mg of ascorbic acid daily, there was a definite early decrease in the amount of phosphomolybdate-reducing substances present in the urine which was probably not due to the change in ascorbic acid intake, since the 5th subject, who

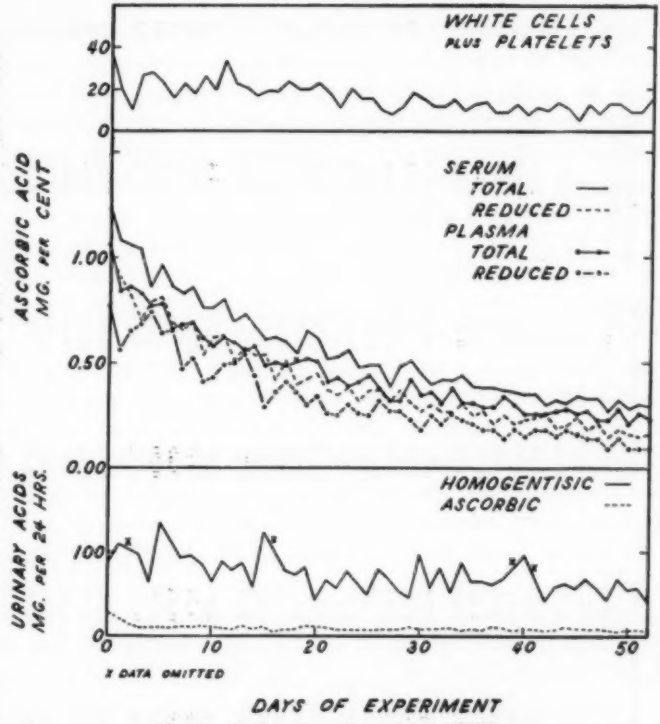

Fig. 1 Daily values for subject MLW.

received 75 mg of ascorbic acid per day, showed a similar trend.

Table 2 includes the data from the 5th subject, who was receiving 75 mg of ascorbic acid daily. It is apparent that, while there was, perhaps, a slight change from the first to the second 10-day period, there was no significant change thereafter in any of the constituents measured.

TABLE 2

Mean values for 10-day intervals

SUBJECT	INTERVAL	SERUM ASCORBIC ACID		PLASMA ASCORBIC ACID		WHITE CELL-PLATELET ASCORBIC ACID	URINARY		WHOLE BLOOD ASCORBIC ACID [1]
		Total	Reduced	Total	Reduced		Ascorbic acid	Homogentisic equivalents	
	days	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg per 24 hrs.*	*mg per 24 hrs.*	*mg %*
BLD	1–10	0.95	0.81	0.78	0.71	24.9	12	84	
	11–20	0.58	0.47	0.52	0.37	20.6	10	78	
	21–30	0.44	0.35	0.36	0.27	13.8	9	59	
	31–40	0.32	0.23	0.27	0.18	13.2	9	66	
	41–50	0.25	0.18	0.22	0.13	11.6	9	55	0.45
MLW	1–10	0.91	0.72	0.73	0.58	21.1	12	95	
	11–20	0.66	0.50	0.54	0.40	21.9	9	79	
	21–30	0.49	0.34	0.39	0.26	14.4	9	66	
	31–40	0.39	0.26	0.32	0.20	11.5	9	70	
	41–50	0.32	0.20	0.26	0.15	10.8	8	56	0.49
HHY	1–10	0.95	0.79	0.74	0.62	24.6	19	106	
	11–20	0.64	0.44	0.52	0.38	19.6	11	64	
	21–30	0.47	0.35	0.37	0.28	13.2	9	56	
	31–40	0.38	0.24	0.30	0.18	13.6	10	62	
	41–50	0.30	0.18	0.23	0.12	14.8	10	57	0.57
CAS	1–10	1.11	0.91	0.89	0.73	24.4	22	118	
	11–20	0.69	0.54	0.58	0.42	18.1	22	108	
	21–30	0.49	0.38	0.39	0.32	12.7	17	115	
	31–40	0.39	0.28	0.30	0.23	10.8	23	126	
	41–50	0.30	0.18	0.25	0.12	11.6	19	106	0.49
BWC [2]	1–10	0.95	0.75	0.73	0.61	21.6	41	118	
	11–20	0.85	0.71	0.70	0.54	26.6	25	96	
	21–30	0.87	0.80	0.66	0.65	19.6	25	75	
	31–40	0.85	0.73	0.64	0.64	16.9	28	80	
	41–50	0.80	0.67	0.62	0.59	21.9	27	59	0.92

[1] Whole blood ascorbic acid values are means of 7 consecutive determinations made on the 46th to 52nd days, inclusive.
[2] This subject received 75 mg of ascorbic acid daily.

DISCUSSION

In all 5 subjects, irrespective of ascorbic acid intake, there was a significant difference between total and reduced ascorbic acid in both serum and plasma. There was also a significant difference between serum and plasma levels of both total and reduced ascorbic acid. Thus it would seem that when comparison of results from various laboratories is made, account must be taken of the method used and of the fraction of the blood analyzed. We have no ready explanation for the difference observed between plasma and serum values. The removal of fibrin in the clotting process would increase the water content of serum by less than 1%, while the discrepancy between serum and plasma ascorbic acid is of the order of 15% of the plasma value.

The results presented above would indicate that a daily intake of 25 mg of ascorbic acid is not enough to maintain plasma or serum ascorbic acid at a generally accepted "good" level; i.e., 0.6–0.8 mg per 100 ml. A daily intake of 75 mg of ascorbic acid maintained levels of 0.84 mg of total ascorbic acid per 100 ml of serum, 0.73 mg of reduced ascorbic acid per 100 ml of serum, 0.66 mg of total ascorbic acid per 100 ml of plasma and 0.61 mg of reduced ascorbic acid per 100 ml of plasma, for one subject. White cell-platelet levels averaged 21.3 mg per 100 ml. It is suggested that these levels are all "good."

There is no evidence from this study to indicate that there was any disturbance in tryosine or phenylalanine metabolism as a result of the lowered ascorbic acid intake. Urinary excretion of homogentisic acid was increased in none of the subjects studied, and the values obtained for the individual who received 75 mg of ascorbic acid daily were no lower than those for the others.

As was previously mentioned, whole blood ascorbic acid was determined daily for the last 7 days of the experiment. Hematocrit readings had been obtained routinely, so it was possible to calculate the concentration of ascorbic acid in the blood cells for this period. Using the values found in

TABLE 3

Comparison of actual ratio of cellular to plasma ascorbic acid with ratio calculated from whole blood ascorbic acid, using Sargent's formula

SUBJECT	WHOLE BLOOD ASCORBIC ACID [1]	PLASMA ASCORBIC ACID [1]	HEMATOCRIT [1]	CELLULAR ASCORBIC ACID	A_{wb} [2]	A_p [2]	A_c [2]	ACTUAL RATIO $\frac{A_c}{A_p}$	CALCULATED RATIO $\frac{A_{wb}+0.28}{A_{wb}-0.17}$
	mg %	*mg %*		*mg %*					
BLD	0.45	0.22	35.7	0.85	0.55	0.24	1.32	5.5	2.2
MLW	0.49	0.24	41.1	0.85	0.61	0.27	1.31	4.9	2.0
HHY	0.57	0.23	38.7	1.10	0.70	0.25	1.70	6.8	1.8
CAS	0.49	0.23	43.1	0.83	0.61	0.25	1.28	5.1	2.0
BWC	0.92	0.65	39.6	1.34	1.14	0.71	2.07	2.9	1.5

[1] Values in first three columns are means for 7 days; all other values are calculated from these.

[2] A_{wb}, A_p, and A_c are ascorbic acid concentrations in milligrams per cent of water in whole blood, plasma, and cells, respectively.

Bodansky ('38) for cellular and plasma water content, it was then possible to calculate ascorbic acid concentrations in terms of their concentration in the water of whole blood, cells and plasma. Sargent's ('47) formula,

$$\frac{A_c}{A_p} = \frac{A_{wb} + 0.28}{A_{wb} - 0.17'}$$

was then applied and compared with the actual ratio of $\frac{A_c}{A_p}$. The results are shown in table 3. It appears that at these very low concentrations of blood ascorbic acid the formula is not applicable. At the higher concentration of subject BWC, the discrepancy between the calculated ratio and the actual ratio is less, although agreement could hardly be called good.

There was no indication that the change in thiamine intake, after 31 days of the experimental period, in any way influenced the metabolism of ascorbic acid as shown by the analyses reported in this paper.

SUMMARY AND CONCLUSIONS

Report is made of a study in which healthy adult women were maintained for 52 days on a controlled diet with a controlled intake of ascorbic acid. Daily measurements were made of total ascorbic acid in serum, plasma and white blood cells plus platelets, of reduced ascorbic acid in serum and plasma, of urinary ascorbic acid and of urinary homogentisic acid equivalents. Total ascorbic acid was measured in whole blood for the last 7 days. Data from this study show that:

1. The concentrations of both total and reduced ascorbic acid were significantly higher in serum than in plasma.
2. The concentration of total ascorbic acid was significantly higher than the concentration of reduced ascorbic acid in both serum and plasma.
3. A daily intake of 25 mg of ascorbic acid was not sufficient to maintain the ascorbic acid of the blood at a "good" level in 4 subjects.

4. A daily intake of 75 mg of ascorbic acid was sufficient to maintain the ascorbic acid of the blood at a "good" level in one subject.

5. There is no evidence, on the basis of homogentisic acid excretion, that a daily intake of 25 mg of ascorbic acid is not sufficient to maintain normal metabolism of tyrosine and phenylalanine.

6. White cell-platelet ascorbic acid levels fell to half or less of initial values in 4 subjects ingesting 25 mg of ascorbic acid daily.

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THE EFFECT OF TESTOSTERONE PROPIONATE ON NIACIN METABOLISM IN RATS

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In experiments previously reported (Beher and Gaebler, '50), the effect of intramuscular injections of testosterone propionate on the excretion of several vitamins and allantoin was studied in the adult bitch. There was a marked decrease in the excretion of N^1-methylnicotinamide and riboflavin, which suggested that niacin and riboflavin might be (1) absorbed in diminished amounts, (2) metabolized in an altered manner, (3) cleared to a lesser degree by the kidney, or (4) used in increased amounts in the synthesis of coenzymes. Increased coenzyme synthesis was not excluded by our finding that the concentration of coenzymes I and II in red blood cells remained constant, for results in liver or kidney tissue might well be different (Axelrod et al., '39). Accordingly, we have studied the effect of testosterone propionate on the concentration of coenzymes I and II in these organs. Inasmuch as the number of animals required was large, rats rather than dogs were chosen for the experiments. It was, therefore, necessary to demonstrate the feasibility of using this species by investigating the effect of testosterone propionate on urinary N^1-methylnicotinamide excretion in the rat.

EXPERIMENTAL

The study consisted of two parts, the first of which dealt with changes in urinary excretion of N^1-methylnicotinamide,

and the second with changes in the concentration and total amount of coenzymes I and II in liver and kidney.

Female Sprague-Dawley rats, 4 months old, were used in all experiments. For the excretion studies, urine was collected daily, utilizing metabolism cages equipped with paraffin-coated conical drain pans. Toluene and acetic acid were used as preservatives. Total nitrogen was determined by a modification of the Kjeldahl method, and N^1-methylnicotinamide by the fluorometric method of Huff and Perlzweig ('47). For the coenzyme studies, animals were sacrificed by decapitation and coenzymes I and II in liver and kidney were determined at once by fluorometric measurement (Robinson, Levitas, Rosen and Perlzweig, '47).

In all experiments, daily records were kept of weight, water intake, and urine volume. All rats consumed exactly 10 gm per day of a diet consisting of whole wheat flour, 67.44%; casein (vitamin-free), 15%; whole milk powder, 9.89%; NaCl, 0.75%; $CaCO_3$, 1.5%; corn oil, 5.33%. To each kilogram of this diet was added 1 gm of haliver oil.[1] Testosterone propionate [2] was injected intramuscularly.

RESULTS

Weight, urine nitrogen, and N^1-methylnicotinamide excretion

Seven experiments, of which the average duration was 30 days, are presented in table 1. Each experiment was conducted on a single rat. While analyses were made daily, only the averages for periods of some length are recorded. The daily dose of testosterone propionate was 0.5 mg during the first three experiments and 6.25 mg during the remaining ones. In all instances, with the exception of experiment 5, there was a sharp increase in weight during the first period of testosterone propionate administration, and a flat-

[1] Parke-Davis.

[2] Testosterone propionate in a sesame oil solution containing 25 mg per milliliter was purchased from the Schering Corporation.

tening of the weight curve during later periods or when injections were stopped. Nitrogen output fell as expected in all experiments. A rebound above the control level during continuance of injections was observed only in experiments 1 and 7, and the increase in nitrogen output after injections were stopped was also small.

The effect of testosterone propionate on excretion of N^1-methylnicotinamide, unlike its effects on weight and nitrogen excretion, varied in a remarkable manner with the dose. In all experiments (table 1) excretion of the niacin metabolite fell at first. The length of the second period in each experiment corresponds with the time during which excretion remained below normal. This averages 11 days in experiments 1 to 3, in which the daily dose was 0.5 mg, and only 5 days in the other 4 experiments with large doses. Moreover, the output returned only to the control level when moderate doses were used, while in experiments 4 to 7, with large doses, excretion not only remained low for a shorter time, but rose to twice the control level during continuance of injections.

Hepatic and renal coenzymes

Inasmuch as the effects of 0.5-mg and of 6.25-mg daily doses of testosterone propionate on excretion of N^1-methylnicotinamide were quite different, the effects of both dose levels on the concentration and total amount of coenzymes I and II in liver and kidney were studied. After a 30-day preliminary period of observation, during which the weight record, food consumption, and general health of the animals were satisfactory, about half of the rats used in each experiment were given injections of testosterone propionate intramuscularly daily for 10 days. At the end of this period both the control animals and the injected ones were sacrificed. Liver and kidney weights, and the concentrations of coenzymes I and II in these organs, were then determined.

The results are shown in table 2. The increase in liver weight was similar at the two dose levels (14% and 15%),

TABLE 1

Effects of moderate and large doses of testosterone propionate on the urinary excretion of N^1-methylnicotinamide by the rat

EXP. NO.	DAYS	TESTOSTERONE PROPIONATE INJECTED	WEIGHT	URINE N	N^1-METHYL-NICOTINAMIDE EXCRETED
		mg/day	*gm*	*mg/day*	*μg/day*
1	1–10	0.0	192	247	607
	11–20	0.5	202	229	369
	21–28	0.5	208	310	468
	29–31	0.0	211	263	550
2	1–6	0.0	181	262	750
	7–12	0.5	192	201	483
	13–18	0.5	202	216	675
3	1–10	0.0	192	240	605
	11–26	0.5	203	227	372
	27–29	0.5	209	224	612
	30–34	0.0	211	272	575
4	1–3	0.00	227	250	713
	4–9	6.25	239	223	527
	10–18	6.25	252	229	879
	19–21	6.25	255	237	1400
	22–27	0.00	254	267	865
5	1–6	0.00	230	265	547
	7–9	6.25	233	212	355
	10–12	6.25	240	194	581
	13–15	6.25	240	203	1012
	16–21	0.00	239	241	934
	22–27	0.00	237	283	624
6	1–6	0.00	227	255	707
	7–12	6.25	238	223	528
	13–22	6.25	253	227	941
	23–24	6.25	254	247	1470
	25–26	6.25	256	248	812
	27–34	0.00	254	265	812
7	1–9	0.00	234	242	761
	10–15	6.25	245	109	650
	16–27	6.25	257	244	1056
	28–29	6.25	262	256	751
	30–39	0.00	263	251	739

while kidney weight increased 17% at the lower and 37% at the higher level. At both dose levels the concentration of coenzymes I and II was unaffected in the kidney and increased in the liver, so that the total content of these coenzymes increased in proportion to organ weight in the case of the kidney, while in the case of the liver it increased 42% at the lower and 64% at the higher dose level.

TABLE 2

Effect of moderate and large doses of testosterone propionate on the concentration and total amount of coenzymes I and II in rat kidney and liver

GROUP	NO. OF RATS	DOSE [1]	ORGAN	CO. I AND II PER GRAM WET WEIGHT	WEIGHT OF ORGAN	TOTAL CO. I AND II IN ORGAN
		mg		*μg*	*gm*	*μg*
Control	12	0	Kidney	573	1.34	768
Injected	12	0.5	Kidney	572	1.57 [2]	899 [2]
Control	12	0	Liver	554	5.22	2892
Injected	14	0.5	Liver	690 [2]	5.96 [2]	4111 [2]
Control	8	0	Kidney	410	1.36	557
Injected	10	6.25	Kidney	426	1.86 [2]	790 [2]
Control	8	0	Liver	426	6.35	2701
Injected	10	6.25	Liver	608 [2]	7.31 [2]	4445 [2]

[1] Injected intramuscularly daily for 10 days.
[2] Significance of increase: P value < 0.001.

DISCUSSION

The increase in coenzyme content of the liver and kidneys shown in table 2 occurred during a 10-day injection period. In the first three experiments shown in table 1, with moderate doses of testosterone propionate, the average decrease in urinary output of N^1-methylnicotinamide during the period of treatment, which averaged 10 days, was 2,570 μg. The equivalent amount of niacin, converted to coenzymes I and II, would equal about 9 times the total increase of these coenzymes in liver and kidneys found in experiments with 0.5-mg doses of testosterone propionate (table 2).

On the other hand, in experiments 4 to 7 (table 1), with large doses of testosterone propionate, the total excretion

of N^1-methylnicotinamide during the first 10 days of administration, calculated from the daily analyses, decreased an average of 695 μg in two experiments and increased an average of 841 μg in the other two, due to the rapid and extensive "rebound" already mentioned. It would, therefore, be unsafe to assume that diminished conversion to N^1-methylnicotinamide provided any of the niacin required for the increase of hepatic and renal coenzymes observed in experiments with large doses of testosterone propionate (table 2). One also cannot postulate that additional niacin became available as the result of catabolic processes brought about by large doses of testosterone propionate, for our weight records and nitrogen data do not support this assumption, and the best known involutionary effects of testosterone propionate are more readily elicited by small doses than by large ones (Ludwig, '50).

SUMMARY

1. Prolonged reduction of N^1-methylnicotinamide excretion was observed in 4-month-old female rats which received 0.5 mg of testosterone propionate intramuscularly daily.

2. When the daily dose of the hormone was increased to 6.25 mg, excretion of the niacin metabolite fell at first, but subsequently rose to about twice the control value.

3. In the kidney, the concentration of coenzymes I and II was unaltered by injecting daily, for 10 days, 0.5-mg or 6.25-mg doses of testosterone propionate, the increase in total amount of the coenzymes being proportional to the increase in organ weight produced by the hormone.

4. In the liver, both dose levels of testosterone propionate increased the concentration of coenzymes I and II, as well as organ weight. The increase in the total amount of these coenzymes in liver was therefore large, being 42% and 64%, respectively, after 0.5-mg and 6.25-mg daily doses.

5. The assumption that diminshed conversion to N^1-methylnicotinamide provided the niacin required for in-

creased synthesis of coenzymes was tenable in some experiments but not in others.

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THE IMPORTANCE OF CHOLINE IN THE PREVENTION OF NUTRITIONAL EDEMA IN RATS FED LOW-PROTEIN DIETS[1]

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THREE FIGURES

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For many years a deficiency of dietary protein has been considered the primary cause of nutritional edema. During and immediately following World War I, when nutritional edema was a major public health problem among undernourished people in the European countries, attempts were made to produce this disease experimentally. Denton and Kohman ('18) produced edema in 4 of 9 rats by feeding a low-protein diet composed principally of carrots. Maver ('20) and Kohman ('20) obtained similar results by using the same diet. Somewhat later Frisch, Mendel and Peters ('29) used similar diets and produced edema in 14 of 30 rats. They also noted that the edema was accompanied by lowered serum protein concentrations. Bloomfield ('33) was unsuccessful in an attempt to produce edema in mature rats on a yeast-alfalfa low-protein diet supplemented with adequate vitamins. In this same experiment, no significant decrease in serum protein concentration was noted aside from an initial drop. He repeated Kohman's work (Bloomfield, '34) and succeeded

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in producing edema when he fed fresh carrots, but failed when he fed dry carrot powder. He adduced that the water in the fresh carrots might be the crucial factor in the production of edema. Recently Dicker, Heller and Hewer ('46) reported the production of nutritional edema in 40 of 68 rats fed low-protein diets.

It is difficult to assess the extent of the edematous condition that was obtained in the above-cited studies. From the description of the condition, however, it must be assumed that extensive accumulations of edematous fluid in the subcutaneous regions rarely occurred, and that the presence of ascites or thoracic fluid served as evidence of edema.

In studies designed to evaluate the importance of dietary choline in the prevention of anemia in rats (Engel, '48), a severe nutritional edema was observed in 7 of 12 adult rats that were fed a low-choline, low-protein diet for 4 to 6 months. The severe edema, anemia and liver cirrhosis that were present in these animals could be prevented by supplying dietary choline. This preliminary observation suggests the importance of choline in the prevention of nutritional edema.

The present study had as its objectives: (1) the development of low-choline diets that would produce severe edema consistently; and (2) a study of the sequence of changes that occurred in the blood hemoglobin, blood serum protein concentration, liver fat content, and total body composition during the edema-induction process.

EXPERIMENTAL

Treatment of weanling animals

Male and female rats of the Alabama Experiment Station (AES) and Sprague-Dawley (SD) strains, aged 23 days and weighing 40 to 60 gm each, were housed individually in screened-bottom cages. Feed and water were supplied ad libitum. Records were kept of food consumption and the rats were weighed weekly. The percentage composition of the diets used is given in table 1.

Procedures and methods

Three procedures have been used to induce nutritional edema. In the first procedure, groups of weanling rats were placed on a diet (C-182) containing 18% of protein and supplemented with 1 gm of choline per kilogram of ration. The

TABLE 1

Percentage composition of the basal diets[1]

INGREDIENTS	DIET		
	C-182	C-189	C-201
Extracted casein[2]	6.0	2.0	3.0
Extracted peanut meal[3]	25.0	10.0	15.0
Salts[4]	4.0	4.0	4.0
Sucrose	44.9	63.9	57.9
Lard	19.0	19.0	19.0
Cod liver oil	1.0	1.0	1.0
L-cystine	0.1	0.1	0.1

[1] Each kilogram of basal diet was fortified with the following: 2 mg each of thiamine and pyridoxine, 4 mg of riboflavin, 10 mg of calcium pantothenate, 20 mg of niacin, 200 mg of *i*-inositol and 50 mg of alpha-tocopherol. Control animals received a further supplement of 2,000 mg of choline chloride per kilogram of diet. The authors are indebted to Merck and Company, Rahway, New Jersey, for a supply of these vitamins.

[2] Commercial casein was purified by percolation with tap water for one day with overnight acidification (0.2% acetic acid) and finally washed with methanol and dried. The purified product contained 87% protein (N × 6.38).

[3] Commercial peanut meal was percolated exhaustively with cold methanol and finally continuously extracted for three days with hot absolute methanol. The extracted material contained 0.05 mg choline per gram and 50% crude protein (N × 6.25).

[4] J. Nutrition, *33:* 155–168, 1947.

amount of choline was reduced in all groups at two-week intervals at the rate of 20% of that present originally until the final choline supplement was zero. During the period of dietary choline reduction, the animals were divided into 4 groups and the amount of protein reduced at subsequent two-week intervals at the rate of 10% of that present originally until the total content was approximately 7% (table 2).

Procedure 2 for inducing nutritional edema consisted of placing weanling rats directly on the choline-deficient diet (C-189) containing 7% of protein. Control animals received the same diet supplemented with 2 gm of choline chloride per kilogram of diet.

In procedure 3, weanling rats were placed on a diet (C-201) containing approximately 10% of protein and supplemented with 2 gm of choline chloride per kilogram of diet. The protein content of the diet remained constant throughout the

TABLE 2

Edema production in rats by gradual reduction of protein and choline levels in the diet. (Procedure 1)[1]

NO. OF RATS PER GROUP	AVE. INIT. BODY WT.	REDUCTION IN PROTEIN BEGUN		FINAL LEVEL OF 7% PROTEIN REACHED		AVE. FINAL BODY WT.	SURVIVAL TIME		NO. WITH GENERALIZED EDEMA
			Ave. body wt.		Ave. body wt.		From weaning	On 7% protein	
	gm	*wk.*	*gm*	*wk.*	*gm*	*gm*	*wk.*	*wk.*	
6	45	2nd	(98)[2]	12th	(198)	145	23	11	5
5	48	4th	(148)	14th	(208)	154	25	11	4
5	46	6th	(158)	16th	(212)	161	27	11	3
6	47	8th	(182)	18th	(212)	165	28	10	6

[1] Initial choline level of 0.1% was gradually reduced to zero after 10 weeks.
[2] Figures in parentheses are the body weights at the times indicated.

experiment, but the choline supplement was reduced at two-week intervals at the rate of 20% of the amount present originally. Control animals were maintained on the same diet supplemented with 2 gm of choline per kilogram of diet throughout the experiment.

Moisture determinations

The rats were weighed, killed by being stunned, and dried in an electric drying oven at 105°C. for at least 48 hours. Moisture weight was considered to be the difference between the live and dry weight.

Fat determinations

Fat determinations were made in some cases on livers alone and in other cases on the total carcass including liver. The dried, pulverized, redried and weighed samples were extracted with anhydrous ether. Fat was expressed as total ether-extractable material.

Protein determinations

Kjeldahl nitrogen determinations were made on aliquots from the moisture-free, fat-free carcasses. The nitrogen values obtained were multiplied by 6.25 to give protein values.

Blood determinations

For hemoglobin determinations, blood samples were obtained by bleeding from the tail. The acid hematin method was used (Engel, '48). Pooled blood serum samples were analyzed by the micro-Kjeldahl method for total protein ($N \times 6.25$; Keys, '40); for albumin (filtrate from 22% sodium sulphate precipitate; Howe, '21); and for non-protein nitrogen (filtrate from 8% trichloracetic acid precipitate; Daly, '33). Globulin was calculated by difference.

RESULTS

Generalized edema was not considered as being present unless the ascites and hydrothorax were accompanied by well-defined pouches of fluid in the subcutaneous tissue. These pouches of fluid were observed to shift from one part of the body to another. The severity of this edema and the shifting of the fluid is illustrated in figures 2 and 3. The accumulation of ascitic fluid, evidenced by abdominal distention, invariably preceded the appearance of generalized subcutaneous edema. The animals seldom survived more than three or 4 days after the appearance of generalized edema. Fatty and cirrhotic livers were always found in the animals with edema.

From the data in tables 2 and 3, it is evident that severe generalized nutritional edema can be induced by either pro-

cedure 1 or 2. The feeding of the 7% protein, choline-deficient diet from weaning (procedure 2) appears to be the preferred procedure, since only 12 to 15 weeks were required to induce the edema (table 3), whereas the initial feeding of higher protein levels (procedure 1) required 23 to 28 weeks (table 2). Moreover the latter procedure necessitates the initial feeding of some choline to prevent deaths from the kidney hemorrhage of acute choline deficiency. Deaths from this cause are seldom encountered when the low-protein, choline-deficient diet is fed from weaning, probably because this diet will permit only very slight body weight gain even when supplemented with choline.

TABLE 3

Edema in choline-deficient rats of two strains fed a 7% protein diet from weaning. (Procedure 2)

STRAIN	NO. OF RATS	AVE. DAILY FOOD CONSUMPTION	AVE. BODY WEIGHT		AVE. SURVIVAL	NO. WITH GENERALIZED EDEMA
			Initial	Final		
		gm	*gm*	*gm*	*wk.*	
SD	14	6.1	52	84	12	12
AES	10	4.5	47	66	15	8
SD[1]	10	4.3	46	76	25	0

[1] Seven per cent protein diet supplemented with 2 gm choline per kilogram diet.

When the 7% protein, choline-deficient diet was supplemented with choline, survival was considerably prolonged (table 3) and the edema was completely prevented. The cause of death in these animals was not established, although evidence of respiratory infection was usually noted at autopsy.

When procedure 3 was used (approximately 10% protein diet) generalized edema was induced in 5 of 6 rats in 17 to 30 weeks, with an average over-all survival period of 23 weeks. Six control animals that received the choline supplement throughout the experiment and were sacrificed three weeks after the last of the choline-deficient animals had died were found to be free of edema.

The number of animals used in these studies was insufficient to warrant any conclusions concerning sex, litter or strain effects. In all these studies, however, about equal numbers of each sex were used and there was no indication of differences in behavior. Whether or not the difference in average survival between rats of the two strains used (table 3) is significant remains to be established.

Preliminary data have been obtained on the body composition of weanling rats fed either an edema-inducing diet (C-189) or the same diet supplemented with choline (table 4).

TABLE 4

Average body composition of rats fed an edema-inducing diet (C-189) with and without choline [1]

(Average of three rats per treatment)

DIET	AVE. INITIAL BODY WT.	AVE. FINAL BODY WT.	AVERAGE BODY COMPOSITION				
			Protein		Fat		Moisture
			Dry basis	Fresh basis	Dry basis	Fresh basis	
	gm	*gm*	%	%	%	%	%
C-189	57	94	11.4	42.8	7.9	29.7	73.4
C-189 + choline	47	66	18.4	55.3	9.9	29.7	66.7

[1] The animals (AES strain) were sacrificed when generalized edema was present in those on the choline-deficient diet.

The rats on diet C-189 had generalized edema when these analyses were made. Rats receiving diet C-189 plus choline did not show indications of ascites or edema of the subcutaneous tissues. The rats with edema showed a substantial increase in body moisture and a resultant diminution in the concentration of body protein and fat when comparison is made with the body composition of rats fed the same diet supplemented with choline. The increase in body weight of the rats receiving this 7% protein, choline-deficient diet over that of the controls receiving a choline supplement can be accounted for almost entirely in moisture.

In another study preliminary data were obtained on serum nitrogen fractions in rats fed either the 7% protein, choline-deficient diet (C-189) or the same diet supplemented with choline (table 5). The 10 rats receiving the choline-deficient diet consistently had about a 25% lower serum non-protein nitrogen and serum protein content than did comparable rats receiving the choline-supplemented diet. The deficient animals all had generalized edema when sacrificed for serum

TABLE 5

Serum protein in rats fed the low protein diet (C-189) with and without choline from weaning[1]

DIET	NO. OF RATS	SERUM NON-PROTEIN-NITROGEN	SERUM PROTEINS		
			Albumin	Globulin	Total
		%	%	%	%
C-189	2	0.107	2.36	0.94	3.30
	4	0.050	2.62	1.07	3.69
	2	0.112	2.59	0.87	3.46
	2	0.075	2.59	1.50	4.09
Ave.		0.084	2.54	1.10	3.64
C-189 + 0.2% choline chloride	4	0.153	3.31	1.20	4.51
	4	0.107	3.18	1.96	5.14
	3	0.104	3.51	1.72	5.23
Ave.		0.121	3.33	1.63	4.96

[1] The animals (SD strain) were sacrificed and blood samples from two to 4 animals pooled when generalized edema had developed in those on the choline-deficient diet.

protein determinations. In this preliminary study no difference in serum protein content due to sex was noted.

Results of a study to determine the change in hemoglobin and liver fat content during the production of edema are given in figure 1. In this study, weanling rats were fed diet C-189 or the same diet supplemented with choline. Hemoglobin determinations were made at two-week intervals on two litter mates, then these animals were sacrificed and liver-fat analyses were made. In rats receiving the choline-deficient diet, hemoglobin decreased from 13.0 gm per 100 ml of blood at

the end of the first two weeks to 4.65 gm at the end of 10 weeks. During this same period, the rats receiving the choline-supplemented diet maintained an average hemoglobin level of approximately 11.0 gm per 100 ml of blood. Liver fat (dry basis) in the choline-deficient animals changed from 74% at the end of the first two weeks to 51% after 10 weeks, while the liver fat in the choline-supplemented rats ranged from

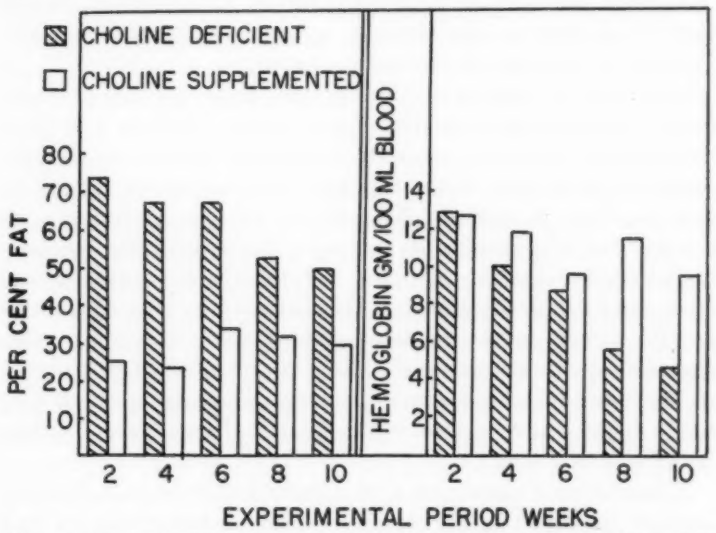

Fig. 1 Average hemoglobin concentration and liver fat of rats fed for varying periods diet C-189 or the same diet supplemented with choline (two rats per treatment per period).

25 to 35% for the same period. These data clearly demonstrate that anemia develops quite rapidly in weanling rats placed on this low-protein, choline-deficient diet. However, it is noteworthy that choline prevented a significant decline in hemoglobin in rats receiving this same low-protein diet.

DISCUSSION

Diets that were sufficiently low in protein to permit only an approximate body weight gain in weanling rats of 20 gm

over a 12- to 15-week period were not edema-inducing so long as dietary choline was supplied. Such diets, however, produced a high incidence of generalized edema when the choline was omitted; choline would thus appear to be more important in the prevention of edema in rats than dietary protein. Schaefer et al. ('51) produced ascites and severe generalized edema in the extremities of dogs that received a choline-deficient diet containing 20% of protein. The latter results further emphasize the greater importance of choline than protein as an edema-preventive agent.

The mode of action of choline may have several explanations. As mentioned earlier in this report, anemia and fatty or cirrhotic livers were consistently found when the edematous condition occurred. Since the liver was damaged, it may be possible that it cannot efficiently carry out its function as a converter of amino acids into new tissue proteins, particularly blood proteins. Support for this theory may be had from data (table 5) showing that edematous rats had about a 25% lower serum protein content than did comparable edema-free rats receiving a choline supplement. The inability of the liver to manufacture sufficient amounts of these proteins could cause many physiological disturbances, edema being one of them.

Schaefer and associates ('51) showed that hepatic function became impaired in chronically choline-deficient dogs 4 to 8 weeks before anemia developed, and that edema did not develop until the animals were severely anemic. Moosnick and associates ('45) reported that human pernicious anemia patients with fatty livers would not respond to purified liver extract unless choline was also administered. Davis and Brown ('47) reported a significant response to choline in two cases resembling Addisonian pernicious anemia that were unresponsive to parenteral liver extracts.

Still another possibility concerning the role of choline in the prevention of anemia must be considered. The diets used in the present study are known to be low in methionine. It is known that choline spares methionine and that methionine

is essential for the formation of blood proteins. Robscheit-Robbins and associates ('47) clearly demonstrated an increased output of blood proteins in protein-depleted dogs resulting from the addition of methionine to an otherwise complete amino acid mixture. Albanese and co-workers ('46) observed a reduction in hemoglobin in rats fed diets nearly devoid of methionine. Li and Freeman ('47) fed rats a diet containing 9% casein as the source of protein and observed a marked growth stimulation and a slight rise in hemoglobin when the diet was supplemented with methionine. Engel ('48) showed that DL-methionine was effective in preventing anemia in rats resulting from a chronic choline deficiency. Jimenez-Diaz and associates ('48) also obtained similar results when they fed casein or other proteins rich in methionine to anemic rats on a choline-deficient diet. Whatever the mode of action of choline may be in the prevention of edema resulting from malnutrition, it is quite clear that anemia and liver damage are abnormalities which appear earlier. It is important to recall that in edema in the human, anemia and liver damage are also generally present. Donhardt and Wodsak ('49) have recently reported the interesting observation that lecithin, given orally, increased water and chloride output and produced striking relief of edema of the extremities in a limited number of patients.

SUMMARY

1. The prolonged feeding of diets low in protein and choline produced severe nutritional edema in 53 of 62 rats. This condition was not observed in control animals receiving the same diets supplemented with choline

2. Rats with edema had a substantial increase in body moisture and a resultant diminution in the concentration of body protein and fat when comparison was made with the body composition of rats fed the same diet supplemented with choline. On a dry weight basis, the rats with edema had low protein values but identical fat values when compared with rats receiving choline.

3. Edematous rats showed a significantly lower serum protein content than rats fed the same diet supplemented with choline.

4. The feeding of a diet deficient in protein and choline caused a rapid development of anemia in rats. In a period of 8 weeks the hemoglobin dropped from 13.0 gm to 4.65 gm per 100 ml of blood. During this same period rats receiving the same diet supplemented with choline maintained an average hemoglobin level of 11.0 gm per 100 ml of blood.

5. The edematous condition was associated with fatty infiltration and cirrhosis of the liver.

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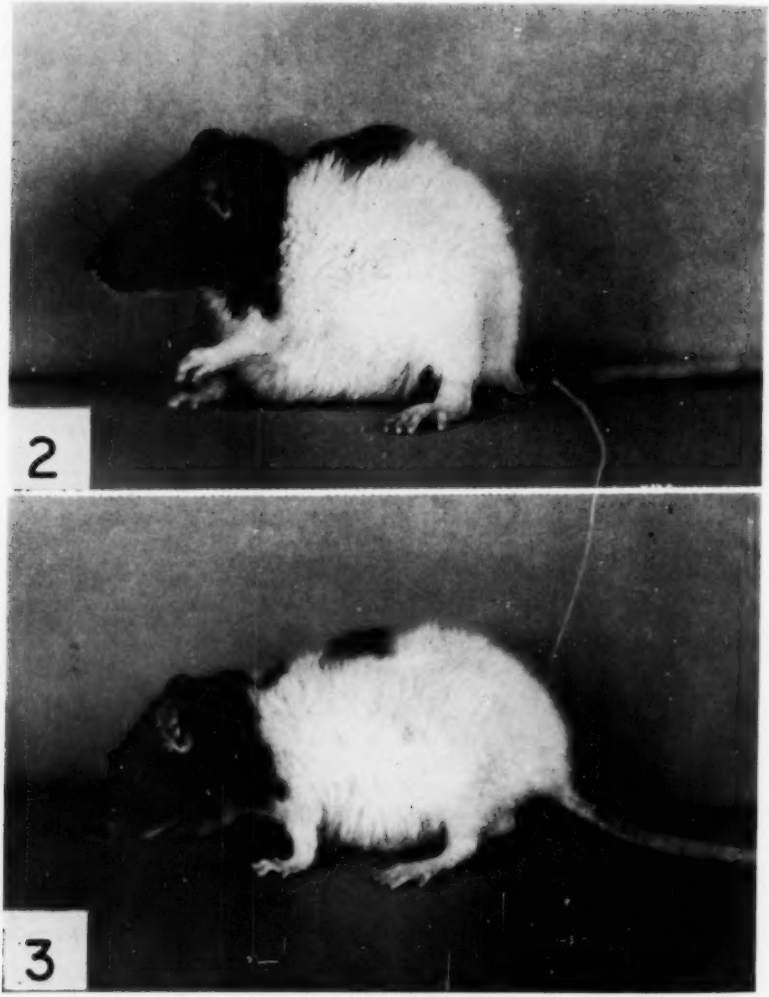

2 Generalized edema in a rat which received diet C-201 for 21 weeks. Note the pouch of fluid between the front legs and extending under the abdominal cavity.

3 Same rat photographed two hours later. Observe the shift of fluid from the subcutaneous tissues.

A STUDY OF LYSINE DEFICIENCY AND NITROGEN BALANCE UNDER AD LIBITUM AND FORCE-FEEDING CONDITIONS [1]

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ONE FIGURE

(Received for publication February 29, 1952)

INTRODUCTION

The nitrogen balance technique of Mitchell ('24) has been used for many years to indicate the retention of nitrogen by animals receiving diets deficient in various amino acids. The results from different laboratories, however, do not always agree. Adult female rats were found by Wolf and Corley ('39) to require lysine to maintain nitrogen equilibrium. Burroughs et al. ('40) found that protein-depleted adult female rats force-fed a lysine-deficient ration did not require lysine to maintain positive nitrogen balance. More recently, however, Frazier et al. ('47) reported that the nitrogen balance of adult male rats force-fed a lysine-deficient ration became negative within about a week. Recent work in this laboratory (Denton, Williams and Elvehjem, '50; Bothwell and Williams, '51) has shown that male weanling rats force-fed a methionine- or histidine-deficient ration may be maintained in positive nitrogen balance even until time of death. These workers noted a constant nitrogen output from rats fed either ad libitum or by force-feeding procedures. When

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the animals received the deficient diets ad libitum there was an immediate decrease in nitrogen intake which rapidly led to a negative balance. It is not difficult to see that a negative balance could be obtained either by an increased excretion of nitrogen or a decreased nitrogen intake. When the nitrogen intake for animals receiving a methionine- or histidine-deficient ration was controlled at a normal level by force feeding, the animals remained in positive balance throughout the experiment, with no tendency for the nitrogen balance to decrease. In all cases, marked correlations between nitrogen intake and nitrogen balance were noted.

The present work was undertaken in order to extend to lysine the results found with methionine and histidine, using the same experimental procedures and methods employed in earlier studies. In this way a direct comparison of the results obtained with deficiencies of these three amino acids can be made.

EXPERIMENTAL

Thirty-two male, weanling rats of the Holtzman strain were divided at random into 4 groups: two groups of 6 rats each (groups I and II), one group of 12 rats (group III) and one group of 8 rats (group IV). Groups I and II were fed ad libitum throughout the experiment, while groups III and IV were force-fed by stomach tube. The rats were placed in metal metabolism cages and fed the purified ration of Ramasarma et al. ('49; see also Denton et al., '50) either ad libitum or by stomach tube (depending on the group) for a week, until they were accustomed to the feeding procedures and were gaining weight. This ration consisted of a purified amino acid mix (16%), salts IV (4%) (Hegsted et al., '41), corn oil (5%), the known vitamins (2%), and sucrose to make up to 100%. All animals were given water ad libitum throughout the experiment. All L-amino acids employed were shown by microbiological assay to be free of lysine.

Groups III and IV were fed by stomach tube in order to negate any effects of inanition due to lowered food in-

take on an amino acid-deficient ration, and to make it possible to compare the results with those obtained with a methionine or histidine deficiency. These groups were fed an amount (about 6 gm per day) previously shown (Bothwell and Williams, '51) to be the average ad libitum consumption of 28 animals fed the complete ration for 7 days. During the pretest period of one week groups III and IV were fed increasing amounts by stomach tube each day until the desired level was reached (7 ml per day). The ration to be force-fed was mixed with water in a Waring Blendor so that 7 ml of this would be equivalent to 6 gm of dry ration. Nitrogen analyses indicated that this mixture contained 16.6 mg N per milliliter. Feedings were made three times daily, spaced 6 hours apart. In order to eliminate as much contamination of the urine and feces as possible, groups I and II were tongue-fed through wire mesh (no. 4 mesh). At the end of the one-week pre-test period, the animals had become accustomed to the ration and feeding procedure and were gaining weight at a good rate.

All urines were collected under toluene acidified with hydrochloric acid. Collections for all groups were made every 4 days, except for the groups receiving the lysine-deficient ration by stomach tube. After the first 4-day collection, the urines for this group were collected every two days. The urine samples were then diluted to 250 ml for nitrogen determinations. Fecal collections were made at the same time as urine collections. The feces were covered with 95% alcohol, acidified with one drop of concentrated hydrochloric acid and dried in a steam oven. The dried samples were weighed and ground in a mortar, and portions were taken for nitrogen determination. A semi-micro Kjeldahl procedure was used for all nitrogen determinations, which were made in duplicate.

RESULTS AND DISCUSSION

The results of the experiment, expressed in weight change, nitrogen intake and nitrogen balance, are presented graphi-

cally in figure 1 (curves I, II, III, IV, V, VI). Table 1 shows the distribution of nitrogen output between urine and feces.

It can be seen from weight curves I and IV that there was an immediate gain of weight for the animals receiving the

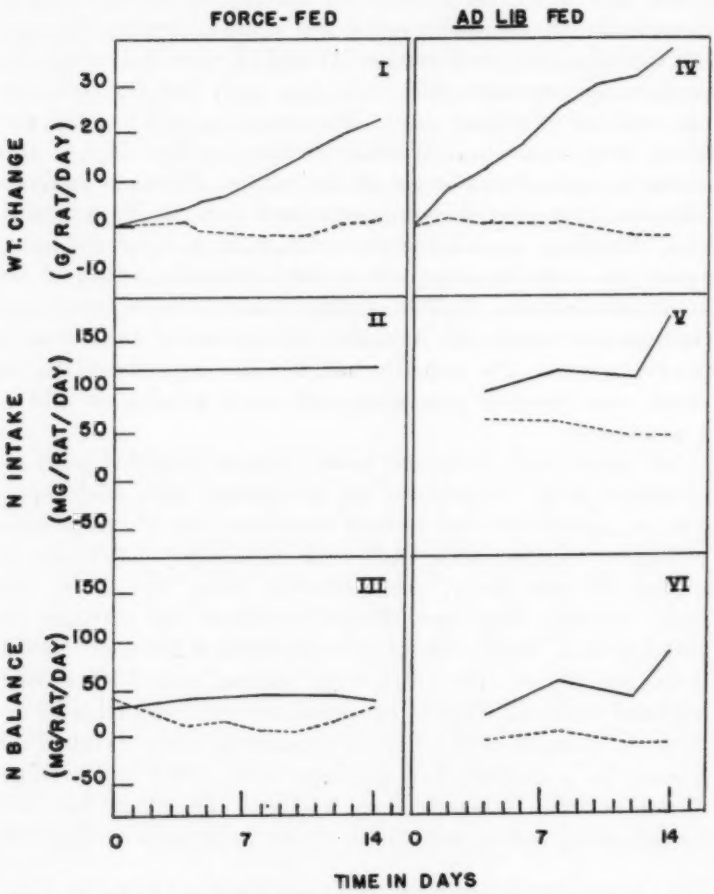

Fig. 1 Weight change, nitrogen intake, and nitrogen balance curves of animals receiving a complete or lysine-free ration ad libitum or by force feeding.
- - - - - - - = lysine-deficient animals
——————— = control animals

complete ration, either ad libitum or by force feeding. The difference in weight gain between the animals receiving the complete ration ad libitum and by stomach tube may be attributable to the amount of ration consumed, because as the animals fed ad libitum grew, they consumed more food, while the groups fed by stomach tube received the same amount daily throughout the experiment. The animals receiving the

TABLE 1

Distribution of nitrogen output for animals fed a complete and lysine-free ration under ad libitum and force-feeding conditions

REGIMEN	PERIOD	NITROGEN OUTPUT	
		Urine	Feces
	days	*mg N/rat/day*	
Complete ration ad libitum	1–4	69	3.5
	5–8	50	4.8
	9–12	61	4.1
	13–14	88	5.3
Deficient ration ad libitum	1–4	69	5.0
	5–8	58	3.0
	9–12	58	4.0 [1]
	13–14	57	2.0
Complete ration force-fed	0	80	4.7
	1–4	73	4.1
	5–8	68	4.6
	9–12	70	3.5
	13–14	79	4.9
Deficient ration force-fed	0	75	4.6
	1–4	101	5.8
	5–6	94	8.7
	7–8	104	10.0
	9–10	106	9.2
	11–12	90	9.0
	13–14	74	4.4

[1] These figures are the averages of fecal nitrogen for the other periods, since fecal samples were lost for these days. The constancy of fecal nitrogen in these groups makes this interpolation possible.

deficient ration by either means gained some weight during the first two days and then slowly lost weight for the remainder of the experiment. These weight changes are somewhat similar to those reported in similar experiments with methionine and histidine deficiencies (Denton, Williams and Elvehjem, '50; Bothwell and Williams, '51).

Curves II and V indicate the nitrogen intake for the 4 groups. The intake for the two groups receiving the complete or deficient ration by stomach tube was constant at 116 mg N per rat per day, while large fluctuations were noted in the intakes of animals receiving the two rations ad libitum. The animals fed the lysine-deficient diet ad libitum evidenced a drop in food consumption, as seen in the decrease of nitrogen intake.

As was noted previously with a methionine- or histidine-deficient diet, curves V and VI show a very close correlation between nitrogen intake and nitrogen balance. A decrease or increase of nitrogen intake was closely followed by a similar decrease or increase of nitrogen balance. Urinary and fecal nitrogen for the groups fed ad libitum varied little throughout the experiment, so it would not be unexpected that the nitrogen balance should vary in close relation to the nitrogen intake. As is shown by curves II and III, this close correlation between nitrogen intake and nitrogen balance did not hold for the animals force-fed the lysine-deficient ration. This is in contrast to the results with methionine and histidine, which showed a close correlation for all groups. It can be seen from table 1 that during the first few days there was an increase of urinary nitrogen from this force-fed deficient group. As the experiment continued and the animals evidenced marked deficiency symptoms, there was a decrease of urinary nitrogen. Thus, the nitrogen balance of the force-fed lysine-deficient group first decreased toward negativity but later became strongly positive as the experiment continued. This is in contrast to the constant urinary excretion and hence constant nitrogen balance found in the histidine and methionine studies for force-fed groups.

These experiments have been repeated in their entirety and very similar results were obtained in both studies. We have reported only one of these complete experiments for the sake of brevity, although both experiments duplicate each other very well.

It appears that a lysine deficiency exerts more of an influence upon urinary nitrogen than do either histidine or methionine deficiencies. Perhaps the increase of urinary nitrogen in the force-fed deficient groups immediately after the animals began to receive the deficient ration by stomach tube is a reflection of their ability to excrete a substantial portion of the unusable nitrogen. Later the marked deficiency symptoms may be correlated with an inability to excrete this excess nitrogen of the incomplete ration, and the animals die while still in positive nitrogen balance.

One important fact that should be considered in interpreting the results of these experiments as well as of the earlier methionine (Denton, Williams and Elvehjem, '50) and histidine (Bothwell and Williams, '51) studies is that the incomplete protein mixture does not appear to be broken down and the "unusable" nitrogen completely excreted. The force-fed animals in the present study appeared to be able to rid themselves of this incomplete protein soon after the beginning of the experiment but, later on, the failure to accomplish this caused a decrease in nitrogen excretion. Even this early increase in nitrogen excretion was not noted in the methionine and histidine studies. The question then arises as to the fate of the incomplete proteins. At the present time investigations are underway by the authors to investigate this problem by studying the nitrogen partition of the organs of the force-fed deficient animals, particularly of the blood.

SUMMARY

The effects of a lysine deficiency upon weight changes, nitrogen intake and nitrogen balance have been investigated, employing both ad libitum and force-feeding procedures.

A marked correlation between nitrogen intake and nitrogen balance was noted for all groups except the lysine-deficient force-fed group. During the first few days of force feeding the latter group showed a marked increase in nitrogen output which decreased as the animals evidenced marked deficiency symptoms.

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THE INFLUENCE OF INGESTED MINERAL OIL UPON THE DEVELOPMENT OF AN ESSENTIAL FATTY ACID DEFICIENCY IN THE RAT [1]

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FIVE FIGURES

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Scientific literature contains many references to the influence of ingested mineral oil upon the absorption of certain of the vitamins. As early as 1927, Dutcher et al. showed that a mixture of mineral oil with butterfat was ineffective in curing vitamin A-deficient rats, and additional work by this group (Dutcher et al., '33, '34) attributed this result to the solvent action of mineral oil on carotene. This effect of mineral oil on carotene utilization has been confirmed with both rats and man by a number of other workers (Alexander et al., '47; Andersen, '38; Collison et al., '29; Curtis and Kline, '39; Curtis and Ballmer, '39; Jackson, '31, '34a; Mahle and Patton, '47; Mitchell, '33; Moore, '29; With, '39, '40, '42; Burns et al., '51).

[1] Portions of the material contained in this paper have been taken from a thesis submitted by E. Kyle Bacon in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry and Nutrition, and from a dissertation submitted by Samuel M. Greenberg in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry and Nutrition, in the Graduate School of the University of Southern California.

Contribution 303 from the Department of Biochemistry and Nutrition, University of Southern California, Los Angeles.

In the case of vitamin A itself (in alcohol or ester form) somewhat conflicting results have been obtained. Moness and Christiansen ('29) could find no difference in the vitamin A activity of a cod liver oil concentrate whether administered in mineral oil or in olive oil. Alexander and associates ('47) likewise failed to show any adverse action of mineral oil on the absorption of vitamin A. Other workers have, however, presented considerable evidence that the absorption of vitamin A is impaired by mineral oil ingestion in both the rat and man, although to a lesser degree than is the case with carotene (Andersen, '39a, b; Curtis and Horton, '40; Hawk et al., '29; Smith and Spector, '40a; With, '39, '40).

Vitamin D has received less attention. The reports of earlier workers (Dutcher et al., '27; Jackson, '34b) showed no adverse action; however, studies by Smith and Spector ('40a, b, c) have indicated that in both rats and dogs mineral oil interferes markedly with the absorption of this vitamin. Elliott et al. ('40) and Lassen et al. ('48) have noted an accelerating action of ingested mineral oil upon the development of a vitamin K deficiency in rats. A similar observation was made by Barnes ('42) with mice, while Javert and Macri ('41) observed the same action in man. No reports have been made concerning the effect of ingested mineral oil on vitamin E, although Smith and Spector ('40a) did report an impairment of fertility in rats receiving 10% mineral oil in the diet. Although these workers did not so conclude, this impairment may have been due to a vitamin E deficiency, since Lassen and associates ('48) found evidence of a possible vitamin E deficiency in adult male rats receiving as little as 5% mineral oil in their ration.

Since the effect of mineral oil upon the absorption of some fat-soluble vitamins seems well established, it was thought of interest to extend these studies to determine whether or not mineral oil also interferes with the absorption of fatty acids. If so, it is conceivable that mineral oil could be used to accelerate the development of an essential fatty acid deficiency. Such a procedure would be highly desirable in view of the long depletion period required at present. Burr and co-workers

were able to produce an essential fatty acid deficiency in weanling rats fed a fat-free diet, in from three to 5 months, with skin symptoms developing in 70 to 90 days but without cessation of weight gain in some cases until the animals were 5 months of age (Brown and Burr, '36; Burr and Burr, '29, '30; Burr et al., '30–'31, '32; Burr and Beber, '37). Other workers have obtained satisfactory depletion in terms of cessation of growth in somewhat shorter periods (Bailey, '43; Martin, '39; McKibbin et al., '39; Quackenbush et al., '39; Quackenbush and Steenbock, '42; Quackenbush et al., '42; Sinclair, '40). Deuel and collaborators ('50) obtained a plateau of weight with male rats after depletion periods of 10 to 12 weeks on a diet very low in fat. Repeated efforts to reduce the last traces of fat in the diet by extraction of the casein, which constituted the only source of fat contamination, failed to reduce the depletion time, indicating that the trace of fat in the diet was not the limiting factor in the appearance of the deficiency symptoms.

The experiments reported below were carried out for the purpose of determining the effect which mineral oil might have on the development of an essential fatty acid deficiency in rats.

METHODS

The composition of the diets is given in table 1. Supplements of the fat-soluble vitamins were given weekly by intraperitoneal injection. In series A to D, vitamins A, D, and E were administered in an ethyl laurate solution and menadione was injected subcutaneously in propylene glycol. All vitamins were given intramuscularly in a propylene glycol solution in series E. The daily dosages were as follows: vitamin A alcohol, 14 μg; vitamin D_2, 0.11 μg; vitamin E, 0.4 mg; and vitamin K (2-methyl-1,4-naphthoquinone), 0.075 mg.

The rats were housed in metabolism cages having wire bottoms so arranged that urine and feces could be collected separately. Analyses were made of the feces only in series A for the first three weeks of the tests. The feces were collected daily and stored in a refrigerator under anhydrous ethyl

TABLE 1

Percentage composition of diets

DIETARY COMPONENTS	DIET							
	1 (basal)	1a	2	3	4	5	6	7
Vitamin-test casein [1]	20.0	20.0 [2]	20.0	20.0	20.0	20.0	20.0	20.0
Sucrose	71.0	71.0	69.0	67.5	66.0	63.5	61.0	68.8
Cellulose [3]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Salt mixture [4]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Water-soluble vitamins [5]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Sulfasuxidine	..	..	..	..	..	..	..	2.0
Streptomycin [6]	..	..	..	..	..	..	..	0.2
Mineral oil	.	..	2.0	3.5	5.0	7.5	10.0	..

[1] General Biochemicals, Inc.

[2] Casein replaced with spray-dried casein hydrolysate and fortified with 0.5% L-tryptophan in diet 1a.

[3] Cellu flour obtained from Chicago Dietetic Supply House.

[4] Osborne-Mendel salt mixture.

[5] The water-soluble vitamin mixture had the following percentage composition: Series A and C — choline and inositol, each 12.0; para-aminobenzoic acid, 6.0; thiamine chloride hydrochloride, 0.72; calcium pantothenate, 0.67; niacin, 0.60; riboflavin and pyridoxine, each 0.27; folic acid, 0.10; biotin, 0.02; and sucrose, 67.6% Series B and D — choline, 12.0; inositol, 5.0; para-aminobenzoic acid, 2.0; riboflavin and thiamine chloride hydrochloride, each 0.72; calcium pantothenate, 0.67; pyridoxine, 0.27; folic acid and niacin, each 0.10; biotin, 0.01%; and sucrose, 78.41%. In series E, the amounts of water-soluble vitamins were double those used in series A and C.

We wish to thank Hoffmann-La Roche, Inc., for the biotin, Lederle Laboratories for the folic acid, and Merck and Co., Inc. for the other synthetic vitamins.

[6] Kindly supplied by Parke-Davis and Company.

alcohol.[2] Water consumption was likewise determined in two experiments (series A and C).

Weanling male albino rats, obtained from our stock colony, were used for the tests. The experiments were continued for as long as 15 weeks in some cases, but in most instances they had a considerably shorter duration. A commercial mineral oil [3] (heavy California liquid petrolatum) was used.

[2] Formula 3A (anhydrous ethyl alcohol denatured with 5% methyl alcohol).

[3] Squibb.

RESULTS

Effect on growth

The results of the 5 series of tests are recorded in table 2. Although there was no shortening of the period required for depletion in essential fatty acids when 2 or 3.5% of mineral oil was incorporated in the diet (groups 2 and 3), the time required was appreciably shortened when the diet contained 5% of mineral oil (group 4). The usual period of 11 to 12 weeks necessary to produce the essential fatty acid deficiency in the present tests was lowered to three to 4 weeks in series A, but it was 6 to 8 weeks in series C (group 11) and 10 to 11 weeks in the case of series E (group 20). It is believed that these variations may be ascribed to variations in the body stores of essential fatty acids at the beginning of the experiment. When the level of mineral oil was increased to 7.5% (diet 5), the period of depletion was approximately 4 weeks (series D, group 14, and series E, group 22). When the mineral oil was increased to 10%, the depletion period had a duration of only two to three weeks.

Figure 1 gives typical growth curves with the several levels of mineral oil (series B), while figure 2 records the results of series E.

Symptoms of deficiency

The symptoms obtained on diets 1, 2, and 3 were typical of those previously observed in essential fatty acid deficiency. Additional symptoms were, however, observed with diets 4, 5, and 6. These were symptoms ordinarily associated with a biotin deficiency. For example, in the case of group 11, in addition to the failure to grow, the rats exhibited an alopecia, particularly on the head, and priapism was present in 9 of the 10 rats in the group. There were likewise brown, crusty skin lesions, particularly on the paws, and a "spectacle eye" condition. These conditions are illustrated in figures 3 and 4.

Another symptom, which was particularly pronounced in the rats on the high levels of mineral oil, was increased water consumption. Hove and Harris ('46) have shown that in-

TABLE 2

The effect of mineral oil in causing depletion of essential fatty acids

SERIES	GROUP NO.	DIET NO.	MINERAL OIL	NUMBER OF RATS	LINOLEATE GIVEN DAILY [1]	CESSATION OF GROWTH	REMARKS
			%		*mg*	*weeks*	
A	1	1	0	10	(20) [2]	11–12	
	2	2	2.0	10	0	11–12	
	3	3	3.5	10	0	11–12	
	4	4	5.0	10	0	3–4 [3]	
B	5	4	5.0	9	0	..	Series B experiments terminated after 4 weeks.
	6	5	7.5	9	0	..	
	7	6	10.0	9	0	2–3 [3]	
	8	1	0	9	0	..	
	9	7	0	9	0	..	Contained sulfasuxidine and streptomycin.
C	10	1	0	10	0	11	
	11	4	5	10	0	6–8 [3]	
	11a [4]	1	0	5	0	..	Resumption of growth.
	11b [4]	1a	0	5	0	..	Resumption of growth.
	12	4	5	10	50 [5]	..	Linoleate completely prevented onset of symptoms.
D	13	1	0	9	0	..	Series D experiments terminated at 7 weeks.
	14	5	7.5	9	0	4 [3]	
	15	5	7.5	9	20 [5]	..	
	16	5	7.5	9	20	..	
	17	5	7.5	9	50 [5]	..	
	18	5	7.5	9	50	..	
E	19	1	0	10	0 (50)	..	Supplement started after 7 weeks.
	20	4	5.0	10	0	10–11 [3]	
	21	4	5.0	10	50	..	Linoleate completely prevented onset of symptoms.
	22	5	7.5	10	0	4–5 [3]	
	23	5	7.5	10	50	..	Linoleate did not completely prevent growth retardation.

[1] Administered orally unless stated otherwise.
[2] Given after depletion to 4 rats, with partial resumption of growth and cure of skin symptoms.
[3] Multiple deficiency symptoms (both essential fatty acid and "biotin").
[4] Group 11, after depletion, divided into two new groups and fed new diets without mineral oil.
[5] Intraperitoneally.

creased water consumption parallels the appearance of external symptoms of fatty acid deficiency in the rat. The amount of water consumed rose sharply when mineral oil levels higher than 2% were included in the diet. Group 4, receiving 5% of

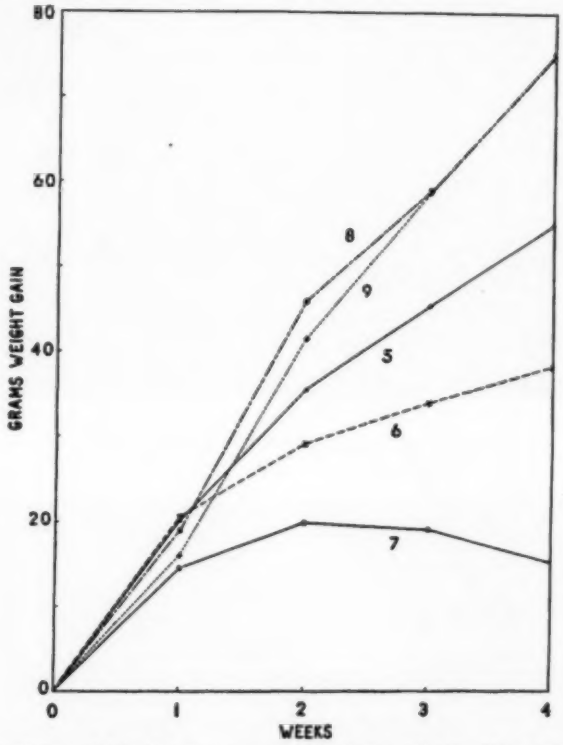

Fig. 1 Mean weight gain of groups of animals in series B. The group number is designated on the chart. No. 5 — 5% mineral oil. No. 6 — 7.5% mineral oil. No. 7 — 10% mineral oil. No. 8 — no mineral oil. No. 9 — no mineral oil; diet contained sulfasuxidine and streptomycin. No linoleate supplement to any group.

mineral oil in the diet, consumed 350 ml of water per day per kilogram body weight at the end of the second week, while at the same time group 3, receiving 3.5% mineral oil, had a water intake of 227 ml per day per kilogram. On the other hand, the

rats on the fat-free basal diet, and those in group 2 on the 2% mineral oil diet, which had not developed deficiency symptoms at this early period, had approximately equal water consumptions of about 160 ml per day per kilogram. A comparison of

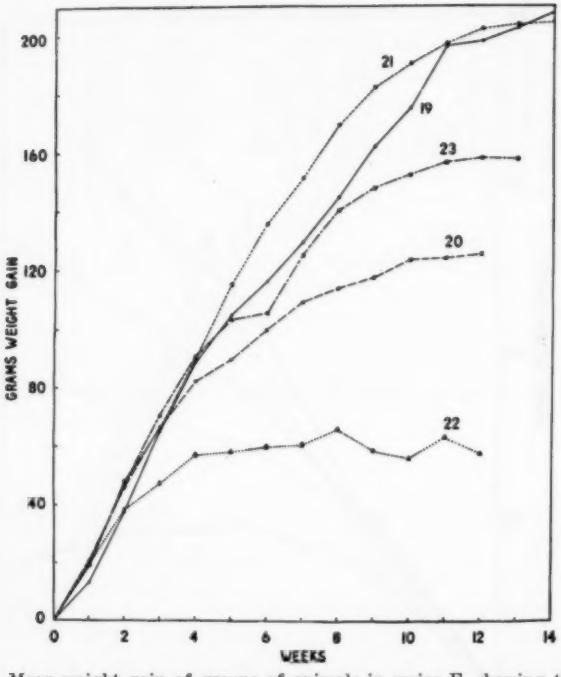

Fig. 2 Mean weight gain of groups of animals in series E, showing the effect of linoleate supplements. The group number is shown on the chart. No. 19 — no mineral oil, linoleate (50 mg) started at the end of 7 weeks. No. 20 — 5% mineral oil in diet. No. 21 — 5% mineral oil in diet, supplemented with 50 mg methyl linoleate per day. No. 22 — 7.5% mineral oil in diet. No. 23 — 7.5% mineral oil in diet, supplemented with 50 mg methyl linoleate per day.

water intake in rats on the fat-low basal diet without and with 5% mineral oil is illustrated in figure 5. The effect of the injection of methyl linoleate on the water consumption of group 12, which also received the 5% mineral oil diet, is also illustrated. Since the food consumption is not significantly changed

Fig. 3 This illustrates the "spectacle eye" condition which occurred in practically all of the rats receiving 5% or more of mineral oil in the diet.

Fig. 4 This photograph illustrates the denuded condition of the skin and the priapism. The brown, crusty skin lesions are not clearly evident except in color.

by the inclusion of mineral oil in the diet, and since the effect on water consumption is reversed by linoleate, this effect seems to be specifically related to the essential fatty acid deficiency.

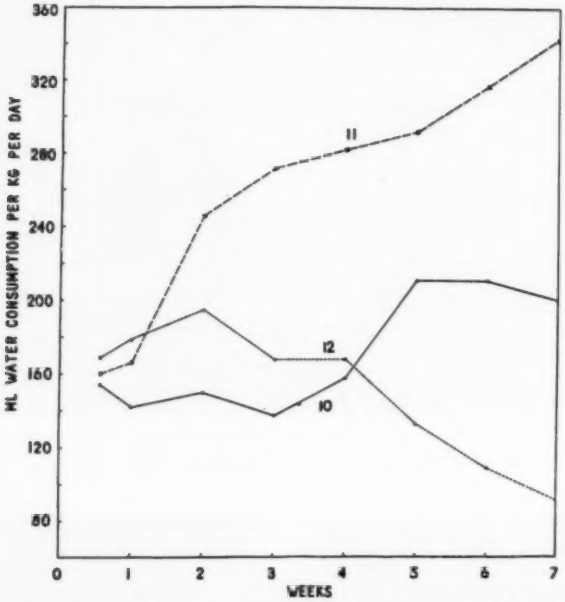

Fig. 5 The average water consumption of male rats (expressed in milliliters per kilogram per day) on fat-low basal diet (group 10), on 5% mineral oil diet (group 11), and on 5% mineral oil diet but also receiving 50 mg methyl linoleate by intraperitoneal injection (group 12) over 12 weeks. Group numbers are indicated on the chart.

Effect of mineral oil on composition of feces

In order to obtain information as to the effect of the inclusion of mineral oil in the diet on the lipid composition of the feces, studies were made of the unsaponifiable fraction, total fatty acids and iodine number of the fatty acids of samples of feces collected during the first three weeks of the experiment in series A.

Composite group samples of the rat feces, representing the total feces excreted by 10 rats over a period of one week, were

dried, ground, and saponified with 0.5 N alcoholic KOH for 30 minutes. The saponified mixture was filtered and the unsaponifiable fraction separated by repeated extractions with petroleum ether (B.P., 30 to 60°C.). The residue was then acidified and the liberated fatty acids were extracted with petroleum

TABLE 3

The weight and composition of the feces obtained from rats on fat-low diets containing 0, 2, 3.5, or 5% of mineral oil

MINERAL OIL IN DIET	WEIGHT OF FECES	WEIGHT OF UNSAPONIFIABLE FRACTION	WEIGHT OF FATTY ACIDS	IODINE NUMBER OF FATTY ACIDS
%	*gm*	*gm*	*gm*	
First week				
0	28.6	0.306	0.263	47.0
2	42.1	9.37	0.660	..
3.5	57.0	14.13	0.570	..
5	66.0	19.25	0.703	55.5
Second week				
0	42.1	0.583	0.436	43.7
2	58.6	11.90	0.657	..
3.5	87.3	..	..	..
5	112.0	33.82	1.719	54.3
Third week				
0	62.5	0.828	0.873	42.6
2	95.8	..	..	..
3.5	114.8	..	..	..
5	149.1	39.08	2.469	46.3

ether. The solvent was removed from both fractions at low temperature under nitrogen and the residues were weighed. The data are recorded in table 3.

DISCUSSION

The results reported in the above experiments demonstrate that mineral oil, added to a fat-low diet such as is usually used to produce an essential fatty acid deficiency, accelerates the development of the deficiency syndrome. This acceleration was particularly noticeable when the fat-low basal diet con-

tained 5% or more of mineral oil, while at levels of 2% and 3.5% mineral oil the effect was less noticeable.

The water consumption, measured in experiments A and C, showed that the groups receiving mineral oil in amounts of 5% had an abnormally high water consumption early in the experiments, before other deficiency symptoms were observed. This suggests that a rapid quantitative method might be worked out, based upon water consumption as a criterion of incipient essential fatty acid deficiency. Such a method would require that due regard be given to the humidity and temperature of the environment.

The results of the analysis of the rat feces in experiment A (table 3) show that, as the level of mineral oil in the fat-low diet is increased, the amount of metabolic fat (expressed as fatty acids) lost with the feces also increases. In addition, the metabolic fat lost with the feces by the group receiving 5% mineral oil in the diet (group 4) was more unsaturated than in the case of the group receiving the fat-low diet (group 1). From this, it appears that one action of the mineral oil is that of removing a greater proportion of unsaturated fatty acids from the rats. However, the results obtained in experiments C and E, using the oral or parenteral route, in preventing the growth-inhibiting action of mineral oil, would indicate that the solvent action of mineral oil cannot provide an adequate explanation of the effect. Furthermore, these results and the failure of sulfasuxidine and streptomycin to speed up the onset of the essential fatty acid deficiency (experiment B) may indicate that no significant amount of essential fatty acids is synthesized by the intestinal flora.

While typical essential fatty acid deficiency symptoms were observed in these experiments with mineral oil, there were other symptoms (the "spectacle eye," alopecia, the humped posture, and the spastic gait) which suggested also a biotin deficiency. An attempt to correct this deficiency by doubling the biotin content of the diet was made in experiment E, and in fact the entire B complex vitamin content was doubled in order to eliminate the possibility of any shortage of the other

B vitamins. This addition did not, however, prevent the appearance of, or alleviate any of, these biotin-like deficiency symptoms. Inasmuch as the amount of biotin given these rats is many times that usually contained in a normal rat diet, we may conclude that they were not biotin deficiency symptoms, or that the mineral oil contained in the diet interferes with the rat's utilization of biotin. That the second of these assumptions is the more probable is indicated by the work of Mackay and Barnes ('41), who reported an apparent sparing action of corn oil fatty acids upon biotin. Our experiment also indicates that there is a relationship between biotin and the essential fatty acids. It has been pointed out by a reviewer that our diets are almost certainly low in B_{12}. This may also have contributed to the development of multiple deficiency symptoms.

SUMMARY

Mineral oil, at levels of 2, 3.5, 5, 7.5 and 10%, was added to a fat-low diet which under ordinary conditions will result in a fatty acid deficiency and cessation of growth in 11 to 12 weeks. Below 5%, mineral oil did not result in cessation of growth earlier than 11 to 12 weeks, but the higher levels of mineral oil resulted in growth failure after a shorter period (two to three weeks on 10% mineral oil). In addition to earlier cessation of growth, other symptoms of fatty acid deficiency appeared, including increased water consumption. The symptoms could be prevented by the inclusion of 50 mg of linoleate per day.

The fecal excretion of total fatty acids was found to be increased by the presence of mineral oil in the diet, and the iodine number of the fecal fatty acids was increased by mineral oil. A purely solvent action of mineral oil apparently cannot be accepted, however, since the deficiency symptoms were prevented by either oral or intraperitoneal linoleate.

In addition to typical symptoms of essential fatty acid deficiency, the animals on diets containing 5% or more mineral oil developed symptoms suggestive of a biotin deficiency. It

was not possible to correct these symptoms by increasing the biotin content of the diet in the absence of essential fatty acids.

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NUTRITIVE VALUE OF LARD AS AFFECTED BY THE PROPORTION OF FAT IN THE DIET

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The proportion of fat in a diet appears to have an important bearing on both the digestibility and the growth-promoting values of certain fats. Hoagland and Snider ('40), in ad libitum feeding tests with diets containing 5, 30 and 55% of 4 types of lard, obtained much better growth in rats when the diets contained 30% of fat. On the other hand, lard was utilized most efficiently at the lowest level of intake. In other experiments, Hoagland and Snider ('41) fed steam-rendered lard at 5, 15, 30 and 54% levels and found that maximum growth resulted when the diets contained 30% of fat and minimum growth when 5% was present.

Forbes et al. ('46a) pair-fed isocaloric quantities of diets containing 2, 5, 10 and 30% of fat, consisting chiefly of lard, to growing rats and found the gains in live weight and the digestibility and retention of nitrogen to be in the order of the increasing fat content of the diets. In a later similar experiment with growing rats on diets containing 2, 10 and 30% of fat, but with increased quantities of vitamins, Forbes et al. ('46b) found that increasing the fat content of the rations produced statistically significant gains of fat and energy and a decrease in heat production. In experiments

[1] Retired.

[2] The authors wish to give credit to Ivan Lindahl and Harry Slofsky of the Bureau staff for determining the gross energy values of diets and carcasses, and for the statistical work, respectively, in connection with the study.

with mature rats on diets containing 2, 10 and 30% of fat and ample supplies of vitamins, Forbes et al. ('46c) found that the fat content of the diet had little effect on the utilization of nitrogen, but that the energy expense of the utilization of isocaloric quantities of the diets decreased as the fat content of the diet increased.

Scheer et al. ('47) determined the relation between the composition of the carcasses of a considerable number of rats and their intake of fat and calories. Diets were fed which contained from 0 to 40% of fat and from 25 to 37.5% of protein. In general, the fat content of the carcasses was not related to the fat content of the diet; rats receiving diets with 20% of fat fed ad libitum had the highest content of body fat.

French et al. ('48) conducted a paired-feeding experiment with two groups of young rats which were fed diets containing 2 and 30% of fat, 7% of protein, and which were otherwise adequate for growth. The experiment lasted 70 days. Decreasing the protein intake from the former 22% level (Forbes et al., '46a, b) to 7% of the diet did not alter the previously reported superior energy utilization of high fat diets by the growing rat. In this experiment increased weight gain, increased body gains of fat and energy, and decreased heat production were associated with the high fat ration.

The study presently reported extends the previous studies of the nutritional value of lard carried on in this laboratory, and was undertaken to obtain further information regarding the effects of adequate diets containing different proportions of lard upon the rate and efficiency of growth of young male albino rats.

EXPERIMENTAL PROCEDURE

The formulas for the diets used in the present experiments are shown in table 1. The casein was a commercial product which was thoroughly extracted with ether. It contained 89.58% of protein (N $\times$ 6.38) and the quantity used in 100 gm of diet 1 and in isocaloric quantities of diets 2 and 3 supplied 20 gm of protein. The salt mixture was prepared as previously described by Hoagland and Snider ('40).

The B vitamins were mixed with dextrose in such quantities that 2.5 gm of the mixture in each diet supplied the following quantities of vitamins: biotin, 0.02 mg; calcium pantothenate, 1.5 mg; choline chloride, 40 mg; folic acid, 0.04 mg; inositol, 20 mg; liver extract, 375 mg; niacin, 5 mg; para-aminobenzoic acid, 7.5 mg; pyridoxine hydrochloride, 0.6 mg; riboflavin, 0.3 mg; and thiamine hydrochloride, 0.3 mg.

The fat-soluble vitamins were mixed with lard in such quantities that 1 gm of the mixture supplied the following quantities to 100 gm of diet 1 and isocaloric quantities of diets 2 and 3: vitamin A, 500 U.S.P. units; vitamin D, 100

TABLE 1

General composition of diets

COMPONENTS	DIET					
	1		2		3	
	gm	*Cal.*	*gm*	*Cal.*	*gm*	*Cal.*
Casein	22.33	120.31	22.33	120.31	22.33	120.31
Salt mixture	4.00		4.00		4.00	
B vitamins in dextrose	2.50	8.45	2.50	8.45	2.50	8.45
Fat-soluble vitamins in lard	1.00	9.43	1.00	9.43	1.00	9.43
Lard	4.00	37.71	9.00	84.84	14.00	131.98
Dextrose (cerelose)	66.17	223.59	52.21	176.42	38.26	129.28
Total	100.00	399.49	91.04	399.45	82.09	399.45

U.S.P. units; alpha-tocopherol, 2 mg; and 2-methyl-naphthoquinone, 0.2 mg.

The lard was rendered in an open steam-jacketed kettle from a mixture of back and leaf fats at a temperature of 240°F. It was filtered and stored in one-pint sealed glass jars at 20°F. Lard was added to the diets in such quantities that it constituted 5% by weight or 11.8% of the energy value of diet 1; 10.98% by weight or 23.6% of the energy value of diet 2; and 18.27% by weight or 35.43% of the energy value of diet 3.

Dextrose (cerelose) $C_6H_{12}O_6 \cdot H_2O$ was used as a source of carbohydrate.

The gross energy values of the constituents of the diets, as determined in a bomb-calorimeter, were as follows: casein, 5.388 Cal.; dextrose, 3.379 Cal.; and lard, 9.427 Cal., per gram. The calculated energy values of the diets were as follows: Diet 1, 3.995 Cal.; diet 2, 4.388 Cal.; and diet 3, 4.866 Cal., per gram. As is indicated in table 1, 100 gm of diet 1, 91.04 gm of diet 2, and 82.09 gm of diet 3 supplied equal quantities of energy. The diets were prepared in batches of 1,000 gm of diet 1 and isocaloric quantities of diets 2 and 3, and stored in covered glass jars at 4° to 5°C.

Thirty-two weanling male albino rats weighing approximately 40 gm each and ranging in age from 20 to 22 days were used in the tests. Three rats were selected from each of 6 litters and 6 rats were chosen from another litter for the feeding tests. One rat selected from each of the same 6 litters and two rats taken from the other litter were killed and analyzed as controls. The 24 rats reserved for the feeding tests were distributed among three groups so that each rat had a litter mate on each of the other two diets. The rats were placed in individual round cages with raised screen bottoms. Feed was supplied in a self-feeder and the bottom of the cage was covered with a sheet of blotting paper. The rats were weighed weekly.

The diets were fed so that the first group received 15 gm of diet 1, the second group 13.66 gm of diet 2, and the third group 12.31 gm of diet 3, these amounts representing isocaloric quantities of the three diets. When all members of each group had consumed practically all the feed offered, isocaloric quantities of the three diets were again placed in the feeders. This procedure was followed throughout the 8-week experiment. The quantity of feed supplied at one time was gradually increased until a maximum of 65 gm of diet 1 and isocaloric quantities of diets 2 and 3 were fed. There was practically no wastage of feed.

The 8 control weanling rats selected at the beginning of the experiment and the 24 litter-mate rats used in the 8-week feeding test were chloroformed and the contents of the diges-

tive tract of each rat were removed. The digestive tract was washed in a stream of cold water and returned to the abdominal cavity. The carcasses were tied to retain the internal organs, wrapped in wax paper and stored in a freezer at approximately — 7°C. until analyzed.

The procedure followed in the preparation of the carcasses for analysis was essentially that described by Leavell ('42). Great care was exercised in grinding and mixing the carcasses so as to secure a uniform product. The moisture, fat and nitrogen content of the carcasses and the energy values of the dried material were determined in duplicate.

RESULTS

The results of the feeding tests with the diets containing 5.0, 10.98, and 18.27% by weight of lard are shown in table 2. Only the mean value for the gain in weight and feed consumption for the 4- and 8-week periods are presented; however, the rats were weighed regularly at weekly intervals. The plan of the experiment provided that the group of 8 rats fed one diet should consume the same quantity of feed, in terms of calories, proteins, vitamins, and minerals, as each group of 8 litter mates fed each of the other two diets. In practice, it was difficult to regulate the feed consumption so exactly but, as is indicated in table 2, the maximum difference between the average intake of the rats on the three diets was only 12 Cal. at the end of 4 weeks and 52 Cal. at the end of 8 weeks.

At the end of 4 weeks there was a maximum difference of only 3 gm among the gains made by the three groups of rats, and at the end of 8 weeks the difference was 10 gm, the smallest gain being made by the rats on the low-fat diet. When the gains in weight are expressed as gain per 100 Cal. of intake, the differences among the gains obtained in feeding the three diets are relatively small. The group fed diet 2 shows the highest value, yet it is only 2.5% greater than the average for diet 1.

TABLE 2

Average gain in weight and feed consumption of groups of 8 male rats fed approximately isocaloric quantities of diets containing different proportions of lard

DIETS	INITIAL WEIGHT	GAIN IN WEIGHT AND FEED CONSUMED IN 4 WEEKS				GAIN IN WEIGHT AND FEED CONSUMED IN 8 WEEKS					
		Gain in weight	Feed consumed	Gross energy consumed	Gain per 100 Cal.	Gain in weight	Feed consumed	Fat consumed	Protein consumed	Gross energy consumed	Gain per 100 Cal.
	gm	*gm*	*gm*	*Cal.*	*gm*	*gm*	*gm*	*gm*	*gm*	*Cal.*	*gm*
Diet 1											
Lard constitutes 5% by weight or 11.8% of energy value	43	135	358.12	1,432.52	9.42	250	859.37	42.97	171.88	3,437.50	7.27
Diet 2											
Lard constitutes 10.98% by weight or 23.62% of energy value	42	138	326.07	1,443.95	9.56	260	794.93	87.31	174.65	3,489.74	7.45
Diet 3											
Lard constitutes 18.27% by weight or 35.43% of energy value	42	136	293.99	1,431.73	9.50	257	716.15	130.84	174.45	3,487.54	7.37

In table 3 are shown data on the mean composition of the carcasses of the 8 weanling rats and the rats fed the three lard diets, including values on the quantities of dry matter, fat, protein and energy stored in the carcasses of the rats on each diet. These values were calculated by determining the means of the differences between the composition of each rat in a group at the end of the test and the composition of the corresponding litter-mate weanling rats at the beginning of the experiment.

The data indicate that the rats on diet 3 contained the most dry matter, fat and gross energy, whereas the rats on diet 2 contained the least of these constituents. The values for the rats on diet 1 occupied an intermediate position. The protein content of the rats on diet 2 was the highest, and these values for the rats on diets 1 and 3 were somewhat lower.

The increase in the dry matter, fat, protein and energy in the carcasses of the three groups of rats at the end of the 8-week experiment indicate that the rats on diet 3, which contained 18.27% of lard, stored the largest quantities of dry matter, fat and energy. The rats on diet 2, which contained 10.98% of lard, stored the least of these constituents, whereas the rats on diet 1 stored intermediate amounts. The rats on diet 2 stored the largest quantity of protein, whereas those on diets 1 and 3 stored practically identical but smaller quantities.

In connection with the storage of fat, protein and energy in the bodies of the rats during the 8-week feeding tests, wide differences were found between the values for individual rats on each of the diets. For example, the following differences are cited: The increase of fat stored by rats on diet 1 ranged from 33 to 80 gm; the increase in protein ranged from 40 to 49 gm; and the increase in energy ranged from 561 to 998 Cal. With the rats on diet 3, increases of fat ranged from 47 to 92 gm; increases of protein from 41 to 53 gm; and increases of energy from 671 to 1,072 Cal.

As regards the gross energy value of the feed retained in the carcasses of the rats at the end of the experiment, those

TABLE 3

Average composition of a group of 8 weanling male rats and three groups of male litter mates fed 8 weeks on approximately isocaloric quantities of diets containing different proportions of lard

DIETS	LIVE WEIGHT	DRY MATTER	FAT	PROTEIN	GROSS ENERGY VALUE	INCREASE IN CONSTITUENTS OF RATS AFTER FEEDING 8 WEEKS				GROSS ENERGY VALUE OF FEED CONSUMED	GROSS ENERGY VALUE OF FEED RETAINED
						Dry matter	Fat	Protein	Gross energy value		
	gm	*gm*	*gm*	*gm*	*Cal.*	*gm*	*gm*	*gm*	*Cal.*	*Cal.*	%
Mother's milk and stock diet	41	10.87	2.77	6.84	63.73						
Diet 1											
Lard constitutes 5% by weight or 11.8% of energy value	293	124.15	64.33	52.27	881.57	113.28	61.57	45.43	817.84	3,437.5	23.75
Diet 2											
Lard constitutes 10.98% by weight or 23.62% of energy value	302	122.08	59.28	54.97	849.87	111.21	56.51	48.12	786.14	3,489.7	22.47
Diet 3											
Lard constitutes 18.27% by weight or 35.43% of energy value	300	131.44	72.14	52.17	948.14	120.57	69.37	45.33	884.41	3,487.5	25.30

on diet 3 showed the highest value and those on diet 2 the lowest value.

Statistical appraisal of results

The analysis of variance method was employed in determining the significance at the 5% level of the data for differences in growth of the rats fed the three diets. The results of the analyses indicate that the observed differences in weight gains, fat and protein increase and retention of gross energy were not significant. Further analysis of the data on weight gains was performed by the multiple covariance method of computing Snedecor's "F" value, in which the results were adjusted to avoid any influence of the variations in initial weight and caloric intake. Also, the significance of differences in weight gains was determined by computing Fisher's "t" values. The results of both of the latter analyses confirmed the conclusion that there were no significant differences in the weight gained by rats fed the three diets.

DISCUSSION

The results of the present experiments are, in some respects, in agreement, but in other respects at variance with the results previously reported by others. For example, Forbes et al. ('46a), in experiments with growing rats fed diets containing 2, 5, 10 and 30% of fat, found that the gains in live weight and digestibility and retention of nitrogen were correlated with the increasing fat content of the diets. In a later experiment with growing rats when the quantities of vitamins were considerably increased, Forbes et al. ('46b) found that increasing the fat content of the diets induced increased body gains of fat and energy and decreased heat production, but the differences in average live weight of the rats which received the different diets were not statistically significant.

In the experiments by French et al. ('48) with young rats on diets containing 2 and 30% of fat and 7% of protein, the differences in the average weights of the two groups of rats

at the end of successive weeks were comparatively small. The differences at the end of 4, 8, and 10 weeks, respectively, were 2, 3, and 5 gm, in favor of the rats on the high-fat diet. The authors state, however, that differences in gain in weight were statistically significant (odds, 50:1). The differences in energy gained in the form of fat were of a larger order, and were also statistically significant (odds, 25:1).

In the present experiments with growing rats fed diets containing 5.0, 10.98, and 18.27% of lard, no statistically significant differences were found in the gains of weight; in the storage of fat, protein or energy; or in the efficiency of utilization of the energy in the feed consumed.

SUMMARY

Modified paired-feeding experiments were conducted with three groups of 8 young male albino rats to determine the effects of isocaloric diets containing 5.0, 10.98, and 18.27% of lard upon gains in weight, fat, protein, and energy, and upon the efficiency of utilization of feed energy.

No statistically significant differences were found in the gains in weight, in the storage of fat, protein or energy, or in the efficiency of the utilization of feed energy as related to the fat content of the diets.

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THE RIBOFLAVIN REQUIREMENT OF THE BABY PIG

I. AT AN ENVIRONMENTAL TEMPERATURE OF 85°F. AND 70% RELATIVE HUMIDITY

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THREE FIGURES

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Only through studies of the nutritional requirements of animals at varied stages of the life cycle and under varied environmental conditions may complete assessment of the nutritional adequacy of diets be obtained. Such studies may also aid in clarifying the physiological role of the nutrients under investigation.

Mitchell et al. ('50) have reviewed the literature on the riboflavin requirement of mammals and have reported the effect of change in environmental temperature on the riboflavin requirement of growing pigs. It was found that at 85°F. the requirement was about 1.2 p.p.m. of air-dry feed, and that at 42°F. it was about 2.3 p.p.m.

The present paper represents an extension of these observations to include baby pigs fed a semi-synthetic milk diet at an environmental temperature of 85°F.

MATERIALS AND METHODS

A total of 42 baby pigs, generously supplied by the Swine Division of this University, was used in the three experiments to be described. Their treatment varied among the experiments, so each will be described separately. All experiments were designed on the premise that animals re-

ceiving rations deficient in riboflavin will gain less well than those receiving adequate rations at the same level of caloric intake. The validity of this principle has been demonstrated by Sure and Dichek ('41), Voris, Black, Swift and French ('42) and Mannering and Elvehjem ('44).

Experiment A

Six Poland China and 12 Duroc Jersey pigs, 4 days old, were caged in groups of 4 or 5 animals in a controlled temperature chamber at 85°F. and 70% relative humidity and fed ad libitum a 30% casein synthetic milk, which is described in table 1. This milk was "complete" except for vitamin B_{12}, which was supplied by weekly injections at the rate of 0.4 μg per day per kilogram of body weight, and riboflavin, which was added to the "milk" at the rate of 0.4 μg per gram of "milk" dry matter. After three days, the pigs were placed in individual cages and fed the same milk ad libitum for 5 days during which time all feed was carefully measured, and the pigs were weighed on alternate days. Sixteen of the pigs were then selected and paired on the bases of size, sex, litter and "thriftness." The latter quality was estimated on the basis of voluntary feed intake and weight gain. With the exception of one pair reserved as negative controls and receiving no riboflavin supplement, one pig of each pair was designated as a positive control and was given 5 μg of riboflavin per gram of "milk" dry matter, while the remaining pig was designated as an experimental animal and was given 1.0 μg of riboflavin per gram of "milk" dry matter. The positive control animals were fed ad libitum and the experimental pigs were fed similarly up to the amount of "milk" consumed by the respective pair mates. All feed offered and refused was carefully measured daily, and the pigs were weighed before morning feedings three times weekly. Rectal temperatures were taken twice weekly, and blood samples from an ear vein were taken at intervals.

The reasoning behind this method of experimentation was that pigs of equal size and consuming equal amounts of a balanced feed should gain at equal rates. If one pig were receiving an amount of riboflavin insufficient to meet its requirement, it was expected that his gain would be less than that of the adequately fed pair mate. This would be an indication of the need for raising the riboflavin concentration

TABLE 1

Composition of synthetic diet

(13.0 or 19.5% dry matter. See text for details.)

CONSTITUENTS	AMOUNT
Casein, "vitamin-free" (%, dry basis)	30
Lard (%, dry basis)	30
Mineral mixture[1] (%, dry basis)	6
Cerelose (%, dry basis)	34
Water-soluble vitamins (mg/liter of "milk"):[2]	
Choline	400
Inositol	40
Ascorbic acid	30
p-Aminobenzoic acid	4.0
Niacin	4.0
Calcium pantothenate	3.0
Thiamine hydrochloride	1.0
Pyridoxine	1.0
Folic acid	0.4
Biotin	0.03
Fat-soluble vitamins (am't/liter of "milk"):[2]	
Vitamin A	2,000 I.U.
Vitamin D	200 I.U.
α-Tocopherol	1.0 mg
2-Methyl-1,4-naphthoquinone	0.26 mg

[1] For composition of the mineral mixture, see Johnson et al. ('48).

[2] Choline, inositol, *p*-aminobenzoic acid, niacin, calcium pantothenate, thiamine hydrochloride, pyridoxine, biotin, riboflavin and α-tocopherol were supplied by Merck and Co., Inc., Rahway, New Jersey, through the courtesy of Dr. Randolph T. Major. Folic acid was generously supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York, through the courtesy of Dr. T. H. Jukes. Ascorbic acid was supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind.

in the feed of the experimental animal. Another criterion, and this proved to be just as useful in the early stages, was the voluntary feed intake, which tended to be less in the experimental animals than in the positive control animals.

During the last week of experiment A and throughout the remaining investigations, the solids content of the "milk" was raised from 13% to 19.5%. This was done in order to facilitate feeding and handling of the "milk," and had no apparent ill effects on the behavior of the pigs.

Experiment B

This trial was conducted in a fashion similar to that of experiment A except that 1.0 μg of riboflavin per gram of dry matter was fed to all pigs during the preliminary period. Ten Duroc Jersey pigs were used.

Experiment C

The final experiment differed from the preceding ones in two respects. Sixteen pigs were used, 11 of Chester White and 5 of Duroc Jersey breeding. The odd one of each breed was used as a negative control. All pigs during the preliminary period received 2.0 μg of riboflavin per gram of "milk" dry matter. This level was chosen as being an estimate of the average requirement based on the preceding experiments. At the start of the test period one pig of each pair was raised to 5.0 μg of riboflavin, and the other one remained on 2.0 μg until this was shown to be adequate; it was judged on the basis of previous experience that three weeks was a sufficient length of time for such a demonstration. Further changes in level of riboflavin intake were made only after the animals had been on a given concentration for a minimum of two weeks. This permitted a more accurate evaluation of the effect of the change than did the more frequent variations employed in the first two experiments. Starting the experimental animals at a presumably adequate

level of riboflavin was advantageous, since it permitted a good start for these small pigs. In experiment C the ideal of equal intake was more accurately maintained by limiting the intake of *either* member of a pair so long as the growth rate of the positive control remained at or above the average for well-fed pigs under these experimental conditions.

Estimates of the riboflavin requirement were made in two ways. At first, visual inspection of the individual growth curves was used. While it was felt that this method gave satisfactory results, the chance for personal error in judgment appeared too great, and effective presentation of the data was difficult. A mathematical approach was finally adopted which yielded more specific information and was free of subjective estimation errors. Since comparison of the growth of pigs of varied size was required, it was obvious that absolute weight gains were a poor basis on which to estimate the effect of varied riboflavin intakes. A more equitable basis of comparison is the gain in weight relative to body size. This we have expressed as the average relative daily gain, calculated on a weekly basis, using the following formula derived from Brody ('45):

$$\text{RDG} = \frac{2.303\ (\text{Log}\ W_2 - \text{Log}\ W_1)}{7},$$

where W_2 equals body weight in grams one week after measurement of body weight, W_1. This method of expressing weight gains is valid only during the self-accelerating phase of growth. The data so obtained and the feed intake data were analyzed statistically by Student's method for paired observations.

Riboflavin analyses were made periodically on the vitamin-free casein used in the rations and uniformly showed no measurable amount of riboflavin present. The samples were prepared for analysis by extraction with acid acetone and analyses were made by use of *L. casei*, as described by Johnson ('48).

RESULTS

In each of the three tests reported, two pigs were employed as negative controls, receiving only the basal diet which contained no measurable amount of riboflavin. In test A one pig died suddenly after 30 days on the deficient diet. The post mortem diagnosis was gastroenteritis. During this period he had gained 3 kg. His pair mate gained 3.5 kg in 6 weeks, then was given 5.0 μg of riboflavin per gram of dietary dry matter. During the next 12 days this animal gained over 5 kg. In test B, both negative controls gained about 3.5 kg in 7 weeks, and 3.0 kg while receiving 5.0 μg of riboflavin per gram of dry diet during the 8th week. In test C, one negative control died at the end of the first experimental week without having shown any weight gain. The remaining negative control gained 1 kg in two weeks, lost the same amount during the next 9 days, and was then given 5.0 μg of riboflavin per gram of "milk" dry matter for the following 5 weeks. During this latter period the pig gained 11 kg in body weight.

The above data show that the basal diet was definitely deficient in riboflavin for baby pigs and that the response of deficient animals to riboflavin supplementation was very marked and rapid. In all cases the increased rate of growth was correlated with increased feed intake.

Marked physical symptoms of riboflavin deficiency were not found in any pigs in these experiments. The deficient animals ate less and grew at a slower rate than those fed adequate amounts of riboflavin. The haircoat of the deficient animals was rougher and the pigs were not so alert as the positive controls. All of these symptoms were reversed by addition of riboflavin to the diet; appetite and alertness were restored rapidly, and improvement in haircoat more gradually.

The average weight gain of the positive control animals in all experiments was 17 kg in 8 weeks, or 0.67 lb. per day.

The data obtained which are relevant to determination of the riboflavin requirement are presented in two tables and one figure.

The pooled data of experiments A and B are shown in figure 1. It may be seen that as the riboflavin in the feed of the experimental pigs was increased up to 2.2 μg per gram, the relative daily gains of these animals approached and finally equalled those of the positive controls. Table 2 shows that the differences between the relative daily gains of ex-

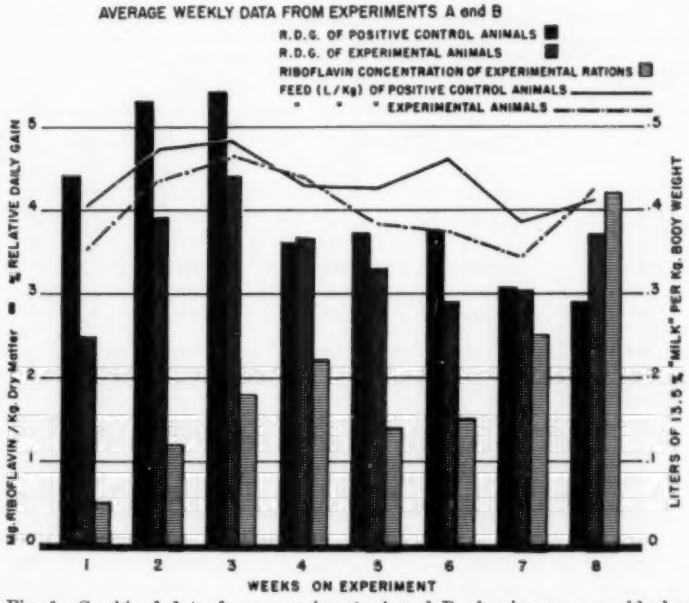

Fig. 1 Combined data from experiments A and B, showing on a weekly basis the per cent relative daily gain (R.D.G.), feed intake, and riboflavin concentration in the ration of experimental animals. The positive control animals received 5 mg of riboflavin per kilogram of dry matter in the ration.

perimental and control animals were highly significant when the former received the 0.5, 1.2 and 1.8 μg levels, but no statistical difference existed when the experimental animals received the 2.2 μg level. During weeks 5 and 6 the average riboflavin intake of the test pigs was 1.4 to 1.5 μg, and the relative daily gain difference was highly significant only in week 6. This indicates that the level of 2.2 μg, fed in week

4, was slightly in excess of the needs of the pigs, and that some slight storage of riboflavin occurred and prevented a marked effect of the deficient intake in week 5. The data obtained in weeks 7 and 8 corroborate the finding of week 4, that the requirement is probably at or below 2.2 μg per

TABLE 2

Statistical treatment of relative daily gain and feed intake data

EXPERIMENTS A AND B	DIFFERENCE IN R.D.G.[1]	"P" (N = 11)	DIFFERENCE IN INTAKE (Liters/kg)	"P" (N = 11)
Week				
1	0.0203	0.01	0.051	0.01
2	0.0134	0.01	0.034	0.12
3	0.0108	0.01	0.017	0.31
4	— 0.0007	0.43	— 0.005	0.45
5	0.0036	0.26	0.040	0.05
6	0.0084	0.01	0.093	0.01
7	— 0.0003	0.45	0.037	0.03
8	— 0.0068	0.04	— 0.010[2]	0.43[2]
EXPERIMENT C		(N = 6)		(N = 6)
Week				
1	— 0.0010	0.44	0.008	0.26[3]
2	0.0022	0.32	0.036	0.03[3]
3	0.0017	0.36	0.032	0.25
4	0.0120	0.01	0.044	0.16
5	0.0212	0.05	0.006	0.45
6	0.0105	0.05	0.051	0.29
7	— 0.0053	0.05	0.022	0.39
8	— 0.0084	0.01	0.043	0.02

[1] Relative daily gain.
[2] N = 6.
[3] N = 7.

gram of feed dry matter. The data of figure 1 are interpreted as indicating a dietary requirement of the baby pig for riboflavin of more than 1.5 and not more than 2.2 μg per gram of feed dry matter at 85°F.

Visual inspection of the pairs of individual growth curves, using parallelism of the curves as the criterion of adequacy, leads to an estimated average requirement for riboflavin of

1.8 ± 0.16 [1] µg per gram, and thus is in agreement with the requirement determined by statistical means.

Experiment C was undertaken to verify the results obtained in the preceding investigations. During the first three weeks, while the experimental animals received 2.0 µg riboflavin, their

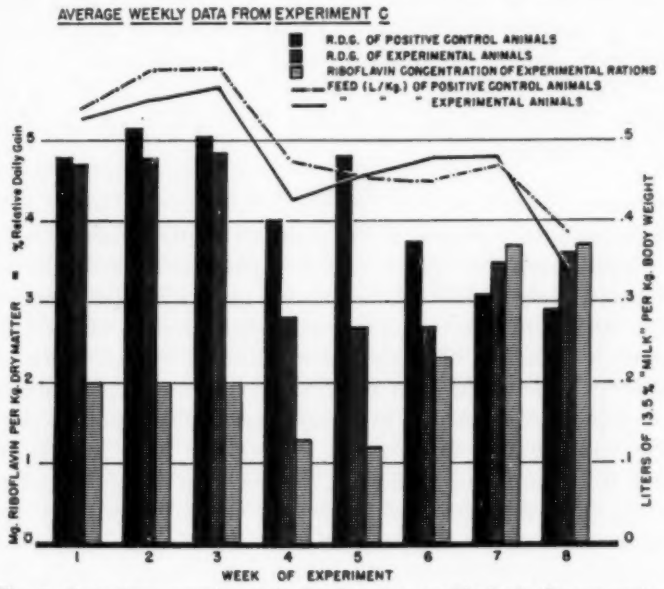
AVERAGE WEEKLY DATA FROM EXPERIMENT C
R.D.G. OF POSITIVE CONTROL ANIMALS
R.D.G. OF EXPERIMENTAL ANIMALS
RIBOFLAVIN CONCENTRATION OF EXPERIMENTAL RATIONS
FEED (L/Kg.) OF POSITIVE CONTROL ANIMALS
" " " EXPERIMENTAL ANIMALS
Mg. RIBOFLAVIN PER Kg. DRY MATTER = % Relative Daily Gain
LITERS OF 13.5 % "MILK" PER Kg. BODY WEIGHT
WEEK OF EXPERIMENT

Fig. 2 Data from experiment C, showing on a weekly basis the per cent relative daily gain (R.D.G.), feed intake, and riboflavin concentration in the ration of experimental animals. The positive control animals received 5 mg of riboflavin per kilogram of dry matter in the ration.

average relative weight gain was statistically similar to that of the positive controls, as is illustrated in table 2 and figure 2. Decrease in the riboflavin level to 1.3 µg resulted in an immediate decrease in rate of gain, demonstrating during weeks 4 and 5 that this level was inadequate. During the following week, three experimental pigs continued to receive only 1.0 µg of riboflavin, and their relative rate of gain aver-

[1] Standard error.

aged 0.0188 ± 0.0031, while one pig continued to receive 2 μg of riboflavin and showed a relative gain of 0.0311; the riboflavin level of the ration given to the three remaining experimental pigs was raised to 5.0 μg at the start of this week and their relative daily gain averaged 0.0285 ± 0.0040.

The results of experiment C are in agreement with those of the preceding experiments. From analysis of the relative daily gain data it is apparent that the requirement of the baby pig for riboflavin, when maintained at 85°F., is not greater than 2.0 μg but is greater than 1.3 μg per gram of feed dry matter.

DISCUSSION

In experiments A and B the positive control pigs were fed ad libitum and the intake of the experimental animals was permitted to equal, but not to exceed, that of the positive controls. Our aim was to maintain equal intakes of feed between the members of each pair of animals by increasing the riboflavin level of the diet when the experimental animal fell behind in feed intake or in weight gain. The application of this principle did not permit equivalent feed intakes between the animals of each pair, as is shown in figure 1. During the first, 5th, 6th and 7th weeks the differences in feed intakes were statistically significant. This makes difficult an interpretation of the significance of the weight gain data in the first three listed periods. In week 7, rates of gain were equal in spite of lower feed intake by the experimental animals than by the controls, and in week 8, when feed intakes per unit of body weight were equal, the rate of gain of the experimental animals actually surpassed that of the controls. In these latter periods, when the experimental animals were receiving adequate levels of riboflavin, the greater rates of gain of the experimental animals may be explained by the fact that they were smaller and thus physiologically younger than the controls; thus, their weight gains were probably higher in protein and water and lower in fat than those of the control animals. Clarke and Smith ('38) reported that

rats restricted in energy intake for short periods exceeded the growth rate of control animals upon realimentation.

Figure 2 illustrates that the equalizing of feed intake was more successfully done in experiment C; hence confidence may be placed in the interpretation that differences in growth rates were due to inadequate levels of riboflavin in the ration of the experimental animals. The data obtained in this experiment corroborate the view that the poor growth of the experimental animals was a result of an insufficient supply of riboflavin, and also illustrate the tendency of rehabilitated animals to grow at a faster relative rate than animals which have always received a balanced diet in similar amount.

Many of the pigs scoured badly in the first experiment. These animals were treated with sulfathalidine, sulfamethazine or streptomycin. The scours were not associated with the level of riboflavin in the ration, and it was routine practice to give the medicine to both pigs of a pair if one of them needed it. That the scours did not seriously hamper the performance of the pigs is shown by the similar behavior of pigs in experiment A and those in experiment C, in which diarrhea was a rare occurrence.

In addition to the two negative control pigs, two others died during the course of these experiments; both animals were from the positive control groups. Autopsy of one animal showed an incarcerated hernia, and of the other, abcessation of the urachus, and nephrosis. Otherwise, except for the diarrhea noted above, the experimental and the positive control pigs were in excellent physical condition throughout the experiments.

Studies were made of the blood hemoglobin, red and white cell counts, and differential counts at intervals during these experiments. None of the data so obtained revealed significant trends that could be correlated with the riboflavin level of the diet. This is in contrast to the earlier investigation in this laboratory (Mitchell et al., '50), in which it was reported that the per cent of neutrophiles in the blood was a sensitive index of the riboflavin adequacy of the diet. The pigs used

in the previous experiment were 8 weeks of age or older at the start of the experiment and their normal percentage of neutrophiles was 14 to 20% of the total leukocytes. The pigs used in the present investigation had an average of 56% neutrophiles at one week of age and 53% at 9 weeks. Different groups of weanling pigs previously used in studies at this laboratory have exhibited averages of 59, 20 and 14% neutrophiles in their blood. The difference between the latter two figures and that obtained on our 9-week-old pigs raised on synthetic milk may be due to variance in diet or some uncontrolled physiologic variable. While it has been reported (Venn, '44) that, in pigs, the per cent neutrophiles is high at birth and low in the adult, the time of this shift, and variables affecting it, apparently have not been thoroughly investigated.

The requirement of the baby pig for riboflavin found in these experiments lies well above 1.3 but not higher than 2.0 μg per gram of diet. This is a definitely higher requirement than that of 1.2 μg reported by Mitchell et al. ('50) for growing pigs maintained at the same environmental temperature of 85°F. The reason for this difference is not certain. It may be based on a less efficient metabolic utilization of riboflavin by the baby pig, or it may be a reflection of increased intestinal synthesis in the older animals. The fact that 85°F. was a comfortable temperature for the baby pigs but appeared to be uncomfortably warm for the larger, fatter pigs may also be involved in accounting for the difference in requirement. A final possibility is that the greater relative rate of gain of the younger pigs required a higher concentration of riboflavin. There are no data to be found in the literature bearing directly on the problem of the relative efficiency of riboflavin utilization between birth and maturity.

Figure 3 shows the change in rectal temperature of the pigs throughout the experiments. Each point on the chart represents the average rectal temperature of the pigs in experiments A, B and C as measured during each week. The probability that the slope of the regression line differs from

zero due to chance alone is less than one in 100. There was no detectable correlation between level of riboflavin intake and rectal temperature.

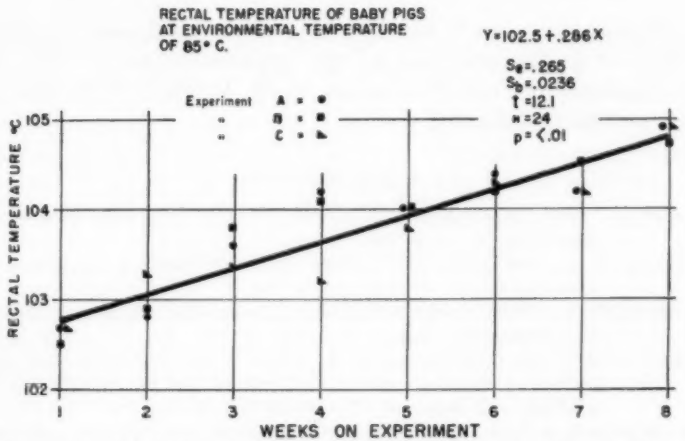

Fig. 3 Combined data of experiments A, B and C, showing regression of rectal temperature (Y) on time (X) of baby pigs maintained at environmental temperature of 85°F. and 70% relative humidity. S_e = standard error of estimate and hence represents amount of variation in rectal temperature not associated with time on experiment. S_b = standard error of b, the slope of the regression line. t = ratio of the slope of the regression line to the standard error of the slope.

SUMMARY

Forty-two baby pigs were utilized in a study of the riboflavin requirement at 85°F. and 70% relative humidity. The basal "synthetic milk" diet was supplemented with varying levels of riboflavin, and the response of pigs so fed was compared with respect to feed intake and weight gain to the behavior of pigs fed the same diet supplemented with an abundant quantity of riboflavin. A modified method of paired feeding for equal intake was utilized. It is concluded that the requirement of the baby pig, maintained under the conditions of this experiment, for riboflavin lies between 1.5 and 2.0 μg per gram of feed dry matter. The rectal temperature

of baby pigs kept under these conditions rose progressively from 102.8°F. to 104.8°F. during the 8 weeks of the experiment.

ACKNOWLEDGMENTS

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THE AMOUNT OF NITROGEN RETAINED BY 6 YOUNG WOMEN ON AN INTAKE OF APPROXIMATELY 70 GM OF PROTEIN A DAY

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In a study in this laboratory of the amount of iron absorbed from spinach (McMillan and Johnston, '51), 6 young women were maintained for a period of three months on a rigidly controlled diet. The study afforded an opportunity to measure the amount of nitrogen retained by normal young women on a constant nitrogen intake over a period of 12 weeks, and to observe the normal fluctuations in the amount retained. The fact that the diet contained no animal protein in the breakfast added interest to the study in light of the recent work concerning the importance of the distribution among the day's meals of the essential amino acids. The amount of nitrogen absorbed from spinach was also noted.

PROCEDURE

Of the 6 subjects, 5 were 20 through 23, and one was 31 years of age. Their mean body size was comparable to that of the population group of 1,661 women from 20 through 24 years of age measured by the Bureau of Home Economics and reported in 1941. The mean height of the subjects of the present study was 159.3 cm (table 1), which was close to the mean of 159.0 cm for the group studied by the Bureau of Home Economics; the mean weight of the subjects of this

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study was less by 2.5 kg than that of the large group. Although all of our subjects were students, they engaged in considerable activity: all were doing laboratory work and had to traverse wide distances to attend their classes.

The subjects were maintained for 12 weeks on an intake nearly constant except for the addition of a serving of spinach

TABLE 1

Body size of the 6 women subjects

SUBJECT	HEIGHT	WEIGHT [1]	SURFACE AREA [2]	
			1st period	2nd period
	cm	*kg*	m^2	m^2
D.D.	156.2	51.2	1.49	1.48
J.S.	153.8	53.9	1.50	1.50
B.M.	160.0	52.1	1.50	1.53
L.O.	160.3	53.5	1.54	1.54
J.M.	162.5	52.9	1.54	1.55
D.C.	163.1	57.0	1.60	1.60
Mean	159.3	53.4	1.53	1.53
Women 20–24 yr.[3]	159.0	55.9		

[1] Mean of the weight at the beginning of each week of the 12 weeks taken before breakfast, wearing no shoes, suit coats or coat sweaters.

[2] The surface area was calculated from the formula, Wt. $^{0.425}_{kg}$ × Ht. $^{0.725}_{cm}$ × 71.84 of Dubois and Dubois ('16). The values used for weight were the means for the weight taken at the beginning of each of the 4 weeks of each period. Weight was taken without shoes, coats or coat sweaters and 0.7 kg was subtracted for clothing.

[3] Values reported by Bureau of Home Economics, U. S. Department of Agriculture, Misc. Publ. 454, 1941. "Women's measurement for garment and pattern construction." The study included 1,661 women 20 to 24 years of age. In the present study the age of all but one of the subjects lay within that range.

each day during the last 8 weeks. The first 4 weeks on the basal diet are designated as period I and the last 8 weeks after the addition of spinach as period II. No preliminary period for adjustment was considered necessary because the experimental diet contained approximately the same amount of nitrogen as the diet upon which the subjects had been living.

The menus, which were made up of a wide variety of common foods, were planned on a weekly basis and repeated 12

times. A serving of beef equivalent to approximately 110 gm of raw beef from which all visible fat had been removed was included in the dinner. All the milk, cheese and eggs were served at the noon meal. An average of 435 gm of milk and 20 gm of cheese per day, and approximately three eggs per week, was used. The breakfast contained no animal protein of any kind, not even milk in the bread. A sample menu is as follows: breakfast — applesauce, cinnamon rolls, coffee; dinner — tomato juice, scalloped potatoes with cheese, coleslaw with bacon, caramel charlotte, coffee and 310 gm of milk; supper — roast beef, rice, gravy, bread, orange juice and coffee. Very little food was allowed ad libitum; extra bread was permitted at the noon meal only and for a given subject the amount consumed per week remained nearly constant throughout the 12 weeks; coffee, tea, a cola drink, butter and sugar were permitted at certain times of the day. The weights of all these foods used by each subject were recorded and analyses were made for those containing nitrogen.

Weekly composites of food, feces and urine were made. The methods of collection and the preparation for analysis of the food and feces were described in detail in a previous paper (McMillan and Johnston, '51). The urine was preserved with HCl without heat treatment. Tampons used for the collection of menses were digested with HCl. The nitrogen content of all samples was determined by use of the Hengar semi-micro Kjeldahl apparatus. The distillation tubes were equipped with jackets to hold ice water to cool the distillate and so prevent loss of ammonia. The amount of nitrogen in the coffee and cola drink was determined and subtracted from the total urinary nitrogen because the nitrogen in these beverages is not available for use by the body.

The energy content of the diet was estimated by weighing each food and calculating the caloric value from values given in the U.S. Department of Agriculture Handbook 8 (Bureau of Human Nutrition and Home Economics, '50).

RESULTS AND DISCUSSION

The mean daily intake of nitrogen on the basal diet in period I was 10.72 ± 0.037 gm. In period II, after the addition of spinach, the intake rose to 11.48 ± 0.064 gm (table 2). The total protein intakes were 67.0 and 71.8 gm in periods I and II, respectively (table 3). Both were above the intake of 60 gm recommended by the Food and Nutrition Board of the National Research Council ('48). On the basis of surface area, the mean nitrogen intakes per day were 7.01 gm per m^2 in period I and 7.49 gm in period II (table 3).

The mean daily loss of nitrogen in the urine was 9.03 gm for period I and 9.85 gm for period II (table 2).

The mean daily loss of nitrogen in the feces was 0.97 gm in period I and 1.04 gm in period II (table 2). The loss was characteristic for a given subject and widely variable from one subject to another.[2] The largest mean daily loss by a single subject for the 8 weeks of the second period was 1.32 gm per day, and the smallest was 0.87 gm. The findings of this study substantiate those of Toscani and Whedon ('51) that direct analysis of fecal nitrogen is indispensable in balance experiments.

The nitrogen content of the menses for 4 menstrual periods was determined for each subject. The mean loss for 6 subjects for 4 periods each was 0.79 gm (table 4). To cover this loss a mean retention of 0.03 gm would be required during every day of the cycle. This loss is so small as to have little importance. However, the loss was probably below the average for all women because the loss of iron, which is correlated with the loss of protein, was below the average (Johnston and McMillan, '52). Also, Barer and Fowler ('36) found the mean loss of blood per menstrual period was 50.5 ml. This amount would contain 1.7 gm of nitrogen if the blood is considered as containing the same percentage as venous blood (3.4%). The amount of nitrogen would probably be somewhat higher than 1.7 gm, as Rona and Waldbauer ('28) found

[2] According to an analysis of variance made on the fecal nitrogen content, F for differences among subjects was 18.04, and according to tables of F, $F_{.05}$ is 5.6.

TABLE 2

The amount of nitrogen ingested, excreted and retained by 6 young women before and after the addition of a 120 gm serving of spinach to the diet

SUBJECT	NITROGEN IN	PERIOD I BASAL DIET					PERIOD II SPINACH DIET									PERIOD I + II
		Week					Week									Mean
		1	2	3	4	Mean	5	6	7	8	9	10	11	12	Mean	
		gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day	gm/day
D.D.	Food	10.87	10.54	10.72	10.57	10.68	11.26	11.27	11.50	11.53	11.56	11.22	11.42	11.66	11.43	11.18
	Feces	0.92	1.40	1.18	1.10	1.15	1.38	1.06	1.14	1.23	0.94	1.38	0.96	1.00	1.14	1.14
	Urine	9.32	8.10	8.77	8.76	8.74	8.92	8.98	9.28	10.40	10.90	10.04	9.88	10.76	9.89	9.51
	Retained	0.63	1.04	0.77	0.71	0.79	0.96	1.23	1.08	— 0.10	— 0.28	— 0.20	0.58	— 0.10	0.40	0.53
J.S.[1]	Food	10.91	10.66	10.76	10.61	10.73	11.26	11.27	11.50	11.57	11.60	11.26	11.46	11.70	11.45	11.21
	Feces	0.94	0.75	0.61	0.84	0.78	1.10	0.86	0.55	0.74	0.90	1.12	0.82	0.84	0.86	0.84
	Urine	9.62	9.59	9.04	9.34	9.40	9.40	9.94	9.76	10.65	10.38	10.03	9.95	9.82	9.99	9.79
	Retained	0.35	0.32	1.11	0.43	0.55	0.76	0.47	1.19	0.18	0.32	0.11	0.69	1.04	0.60	0.58
B.M.[1]	Food	10.98	10.64	10.92	10.48	10.75	11.37	11.42	11.64	11.68	11.78	11.44	11.54	11.86	11.59	11.31
	Feces	1.06	1.20	1.01	0.86	1.03	1.18	1.06	1.11	1.20	0.88	1.00	0.93	0.98	1.04	1.04
	Urine	9.16	8.82	9.24	8.66	8.97	9.43	8.76	9.50	9.44	10.17	9.36	10.32	10.06	9.63	9.41
	Retained	0.76	0.62	0.67	0.96	0.75	0.76	1.60	1.03	1.04	0.73	1.08	0.29	0.82	0.92	0.86
L.O.	Food	10.94	10.66	10.85	10.58	10.75	11.38	11.36	11.62	11.62	11.62	11.38	11.48	11.72	11.52	11.26
	Feces	0.84	0.71	0.74	0.78	0.76	0.93	0.98	0.77	0.92	0.84	0.89	1.03	1.06	0.92	0.87
	Urine	7.74	8.47	9.56	9.06	8.70	9.56	9.11	10.52	10.04	10.31	9.86	10.28	10.16	9.98	9.55
	Retained	2.36	1.48	0.55	0.74	1.28	0.89	1.27	0.33	0.66	0.47	0.63	0.17	0.50	0.62	0.84
J.M.[1]	Food	10.87	10.54	10.73	10.57	10.67	11.26	11.27	11.50	11.53	11.56	11.22	11.42	11.66	11.43	11.18
	Feces	0.98	0.82	0.93	0.84	0.89	0.93	1.14	1.00	0.82	1.10	0.92	0.90	1.00	0.98	0.95
	Urine	9.86	9.48	9.38	9.14	9.46	9.87	9.68	9.82	10.05	10.51	9.96	10.10	9.87	9.98	9.81
	Retained	0.03	0.24	0.42	0.59	0.32	0.46	0.45	0.68	0.66	— 0.05	0.34	0.42	0.79	0.47	0.42
D.C.	Food	10.89	10.62	10.80	10.64	10.73	11.29	11.34	11.61	11.60	11.64	11.30	11.48	11.74	11.50	11.24
	Feces	1.26	1.22	1.16	1.18	1.20	1.37	1.29	1.40	1.34	1.30	1.22	1.27	1.32	1.31	1.28
	Urine	8.68	8.96	8.78	9.14	8.89	9.48	9.73	9.90	9.44	9.70	9.48	9.48	9.68	9.61	9.37
	Retained	0.95	0.44	0.86	0.32	0.64	0.44	0.32	0.31	0.82	0.64	0.60	0.73	0.74	0.58	0.59
All subjects	Food					10.72									11.48	11.23
	Feces					0.96									1.04	1.02
	Urine					9.02									9.84	9.57
	Retained	0.84	0.69	0.73	0.63	0.72	0.71	0.89	0.77	0.54	0.30	0.43	0.48	0.63	0.60	0.64

[1] These subjects received spinach at breakfast in weeks 5, 6, 7, and 8 and for dinner in weeks 9, 10, 11, and 12; the other subjects ate spinach in reverse order.

that most nitrogenous fractions of menstrual blood contain slightly more nitrogen than venous blood.

The mean daily amount of nitrogen retained was 0.72 gm for period I and 0.60 gm for period II, with a mean of 0.64 for the entire 12 weeks (table 2). If the menstrual losses were included these values were 0.03 gm lower. The mean daily

TABLE 3

The amount of nitrogen ingested and retained, the caloric intake per square meter of surface area, and the total protein intake of the 6 subjects

SUBJECT	PERIOD	INTAKE			RETENTION [1]	
		Total protein	N per m^2	Calories per m^2/day	N per m^2	% of intake
		gm/day	*gm/day*		*gm/day*	
D.D.	I	66.7	7.17	1,350	0.53	7.4
	II	71.4	7.72	1,410	0.27	3.5
J.S.	I	67.1	7.16	1,570	0.37	5.2
	II	71.6	7.64	1,630	0.40	5.2
B.M.	I	67.2	7.12	1,580	0.50	7.0
	II	72.5	7.60	1,630	0.60	7.9
L.O.	I	67.3	6.99	1,410	0.83	11.9
	II	72.0	7.46	1,460	0.40	5.4
J.M.	I	66.7	6.93	1,310	0.20	2.9
	II	71.4	7.38	1,350	0.30	4.1
D.C.	I	67.1	6.71	1,350	0.40	5.9
	II	71.9	7.16	1,400	0.36	5.0
Mean	I	67.0	7.01	1,430	0.47	6.7
	II	71.8	7.49	1,480	0.39	5.2
Mean, 12 weeks		70.2	7.33	1,460	0.42	5.7

[1] Menstrual losses have not been deducted.

amount of nitrogen retained per square meter of surface area was 0.47 gm in period I and 0.39 gm in period II (table 3). This was 6.7 and 5.2%, respectively, of the two levels of intake. These values would be lowered slightly by the inclusion of the nitrogen lost in the menses. The mean amounts of nitrogen retained during the 12 weeks (table 2) were similar for all 6 subjects. However, the amount retained varied

widely from week to week in the case of individual subjects. Subject D.D. retained nitrogen during the first 7 weeks and then lost nitrogen during the rest of the time, except for the 11th week. There was no accompanying weight loss and no decrease in caloric intake. Whether there was an increase in energy outgo is not known, as no record of activity was kept. The large amount of nitrogen retained by subject L.O. in the first week of the experiment is puzzling because she had not been living on a low protein diet previous to the beginning of the experiment, according to her diet history. She absorbed more iron and calcium also during the first 4-week period than any other subject.

TABLE 4

The menstrual losses of nitrogen

SUBJECT	NITROGEN LOST IN MENSTRUAL PERIODS				MEAN LOSS PER PERIOD	MEAN CYCLE LENGTH	DAILY RETENTION TO COVER LOSS
	I	II	III	IV			
	gm	*gm*	*gm*	*gm*	*gm*	*days*	*gm*
D.D.	0.64	0.30	0.45	0.88	0.57	23	0.02
J.S.	1.09	0.74	1.44	1.63	1.22	28	0.04
B.M.	0.71	1.21	0.80	0.86	0.90	25	0.04
L.O.	0.58	0.79	0.87	0.76	0.75	31	0.02
J.M.	0.49	0.44	0.53	0.27	0.43	39	0.01
D.C.	0.98	0.88	0.97	0.71	0.88	29	0.03
Mean					0.79	29	0.03

The mean daily amount of nitrogen retained by the 6 subjects dropped during weeks 8, 9, 10 and 11 (table 2). This might be interpreted as meaning that the stores had been filled in the first 7 weeks and that thereafter less nitrogen was retained. This is possible but not likely. Inspection of the values for each individual shows that the negative values for subject D.D. were a large factor in lowering the mean; also, in week 12, the amount retained rose again. Besides, subject D.C., who showed the most consistent behavior with respect to the iron and calcium balances and may therefore be considered the most reliable single indicator, showed no lowering of the amount retained.

The retention of 0.64 gm or approximately 6% of nitrogen per day on an intake of approximately 70 gm of protein (11.2 gm of nitrogen) by the subjects of this study over a 12-week period is approximately the same as the amount observed by others who have studied young women on similar intakes and consuming self-selected diets presumably similar to ones upon which they had been living for a long period of time. Wang et al.[3] ('30) found that approximately 8% of the nitrogen was retained by 6 young women on self-selected diets containing 11.5 gm of nitrogen. McKay et al ('42) studied 41 young women on self-selected diets containing a mean of 10.82 gm of nitrogen, who retained 1.08 gm or 10%.

The mean daily caloric intake for the 6 subjects was 2,180 for the first period and 2,270 for the second; the mean daily intake per square meter of surface area was 1,430 and 1,480 (table 3) for the corresponding periods. The increase in caloric intake in the second period was due chiefly to the addition of spinach and the butter accompanying it. If the energy expenditure of a given subject varied sufficiently from day to day to affect the adequacy of the caloric intake, this could have been one cause of the fluctuation in weekly nitrogen retention. The mean increase of 90 cal. per day during the last 8 weeks did not cause an increase in the amount of nitrogen retained. One way to judge whether the mean caloric intake for the entire 12 weeks was adequate was by body weight. Four of the subjects maintained their weight and two subjects, B.M. and J.M., each gained approximately 3 lb. One of these subjects retained the most nitrogen and the other the least.

Research on animals has shown that the body needs to have all the essential amino acids present simultaneously and in adequate amounts. Research on young women by Leverton and Gram ('49) demonstrated that retention of nitrogen was reduced from 0.96 to — 0.03 gm when all the animal protein in the breakfast was shifted to another meal. In the present study, in which no animal protein was included

[3] The nitrogen in the feces was not determined. It was assumed to be 10%.

in the breakfast, 0.64 gm of nitrogen was retained. Whether the subjects would have retained more had they been placed on a diet with a better distribution of the animal protein is not known, but a retention of 0.64 gm is satisfactory in the light of present knowledge. Possibly these subjects retained a satisfactory amount because the diet contained 1.45 gm more nitrogen than the one employed by Leverton and Gram, or was higher in its energy content, or both. This is logical reasoning because Leverton, Gram and Chaloupka ('51) found that the absence of animal protein in the noon meal did not significantly increase the excretion of nitrogen in the urine unless the intake of energy or protein was low.

This experiment, also, offered an opportunity to observe the amount of nitrogen absorbed from cooked spinach, since the basal diet provided enough solids and roughage so that the small increase from the spinach probably did not raise appreciably the amount of metabolic nitrogen entering the digestive tract. The mean increase in intake of nitrogen per subject per 4-week period after the addition of spinach to the diet was 21.4 gm, of which nearly all (97%) came from the spinach. The resulting increase in fecal nitrogen was statistically significant at the 0.05 level of probability.[4] The mean increase was 2.0 gm. The amount absorbed (the increase in nitrogen intake minus the increase in fecal nitrogen) from the increased intake was 19.5 gm or 91.1%, with a standard error of 4.6%.[5]

SUMMARY

Six young women 20 through 31 years of age were maintained for 12 weeks on a diet which was constant except for the addition of a serving of spinach a day in the last 8 weeks. The nitrogen content of the urine, feces, menses and duplicates of the food was determined.

[4] The "t" value for the difference between the nitrogen content of the feces on the basal diet and on the diet containing spinach was 2.71, and according to tables of t, $t_{.01}$ is 3.36 and $t_{.05}$ is 2.02.

[5] The relative standard error was calculated from the standard deviation for the difference between the nitrogen content of food minus feces on the basal diet and on the diet containing spinach.

The mean intake of protein on the basal diet was 67.0 gm, on the diet containing spinach 71.8 gm, and on both diets for all 12 weeks 70.2 gm. The mean amount of nitrogen retained by the 6 subjects during 12 weeks was 0.64 gm per day, or 0.61 gm if the loss of nitrogen in the menses was deducted. The amount retained varied widely from week to week for a given subject but the means for 12 weeks were similar for all subjects. A satisfactory amount of nitrogen was retained despite the omission of all animal protein from the breakfast.

Approximately 91% (S.E., 4.6%) of the nitrogen of spinach was absorbed.

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NITROGEN BALANCE OF ADULT RATS FED DIETS LOW IN L-, DL- AND D-PHENYLALANINE[1]

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ONE FIGURE

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The influence of low concentrations of dietary phenylalanine on the nitrogen balance of the adult animal has not been extensively studied. Benditt et al. ('50) and Rose ('49) have reported such observations on the rat and the human being, respectively. The latter investigator suggests a tentative minimum daily requirement of L-phenylalanine and states, without giving details, that D-phenylalanine is ". . . partially utilized . . ." by man. Benditt et al. used only DL-phenylalanine in their amino acid mixtures. The work described below was designed to supplement the meager knowledge now available on this subject.

METHODS

Two groups of adult male albino rats, series 260 and 300, were used. At the beginning, and after a 48-hour fast with water given ad libitum, the body weights of the 16 animals of series 260 ranged from 212 to 268 gm (mean = 240 gm); the weights of the 16 animals of series 300 under similar conditions ranged from 248 to 270 gm (mean = 258 gm). The latter group were animals obtained directly from The Wistar Institute of Anatomy and Biology. The others were raised

[1] This investigation was supported in part by a grant from the Office of Naval Research, NR contract N6ori-126 Task IX.

in the laboratory colony from Wistar stock obtained before the last war. The energy allowance for each rat in series 260 was 41.5 Cal./day, and in series 300 it was 43.8 Cal./day (Cal./rat/day = 121 $kg^{3/4}$).[2] Each experiment included the following sequence of feedings: maintenance diet (9.6% whole egg protein), two weeks; nitrogen-free (N-free) diet, one week; amino acid diet containing approximately half of the maintenance requirement of total nitrogen (half-N), one week; amino acid diet containing twice this amount of total nitrogen (full-N), one week. This 5-week cycle of feeding was repeated for each of the rations investigated.

The amino acid mixture employed simulates the proteins of whole egg in that it contains approximately the same amount of each natural isomer of the essential amino acids per gram of total nitrogen. The non-essential amino acids of egg protein are replaced, in this mixture, by the unnatural isomers of 6 essential amino acids plus sufficient L-glutamic acid to make up the same amount of total nitrogen. It is relevant to note that no tyrosine is added. This "complete" amino acid mixture and the basal diet in which it is used are described elsewhere (Anderson and Nasset, '48; Nasset, Anderson and Siliciano, '51).

The N-free diet was compounded simply by omitting the amino acid mixture and substituting an equal weight of sucrose.

It is the custom in this laboratory to observe changes in nitrogen balance which are brought about by a stepwise reduction of the concentration of one essential amino acid at a time. This process simulates the consecutive testing of various proteins which may have different biological values. It is probably a much more physiological procedure than omitting completely one of the essential amino acids from the mixture. At some point in this process a concentration is reached

[2] This formula Cal./rat/day = 121 $kg^{3/4}$ is an empirical relationship derived from our experience with this technique. When our animals are fed according to this formula, the body weight remains constant within 5% for as long as 6 months.

which becomes limiting for the utilization of the total dietary nitrogen. In the present investigation one-third, one-fourth, and one-eighth DL-, one-fourth, one-sixth, and one-eighth L-, and one-eighth D-phenylalanine amino acid mixtures were used. The fractions refer to the total amounts of phenylalanine present, regardless of isomeric form, as compared with the "complete" amino acid mixture. One-eighth D-phenylalanine, for example, simply refers to a mixture which contains one-eighth as much phenylalanine nitrogen as the complete mixture, and identifies the source as the unnatural isomer.

The N-free, half-N and full-N diets were fed by stomach tube in two equal portions daily. The maintenance diet was weighed out accurately each day in feeding cups to provide each animal with a constant intake of protein and energy. This portion was almost invariably eagerly and completely consumed. Total N was determined on feed and excreta by the Kjeldahl method. Each diet period lasted 7 days and feces were collected for the entire period. They were marked by adding Cr_2O_3 to the first feeding of each period. The urine was collected for analysis only during the last 4 days of each period of amino acid feeding.

The determination of nitrogen balance on N-free diets has been abandoned. As has been shown in previous papers (Nasset and Anderson, '50; Nasset et al., '51), the results obtained, under the conditions described above, leave considerable doubt as to their validity as a measure of "endogenous" N metabolism. The N-free periods are retained in the feeding schedule, however, as a device for depleting protein stores just prior to feeding the amino acid mixtures. Other details of the experimental procedure employed are described in an earlier paper (Nasset and Anderson, '50).

RESULTS

Table 1 contains the average results of all experiments in which the phenylalanine concentration was critically reduced. The discovery of a limiting concentration may re-

quire considerable preliminary work. The first guess is usually made on the basis of data taken from the literature, but this technique invariably yields estimates which are too high. It becomes necessary, therefore, at this point to reduce stepwise still further the concentration of the amino acid under study. Series 260 rats were first fed a mixture containing one-third as much phenylalanine as is found in the complete mixture. Since this was not limiting, the following mixtures were given: one-fourth L-, one-sixth L-, "complete," one-fourth DL-, and one-eighth L-phenylalanine, in that order. Thus these rats were on experiment for 6 periods of 5 weeks each. Only the last experiment — i.e., the one in which one-eighth L-phenylalanine was fed — indicated an impairment of nitrogen utilization which could be attributed to reduction in phenylalanine. In order to save space, only these results from series 260 are included in table 1.

Experience with the methods described above suggests that more than one criterion should be used in deciding whether a given reduction in one essential amino acid limits the utilization of the others. First of all, the value of K′ (see table 1 for definition) should be significantly less than unity; as a rule of thumb this usually means 0.8 or less. If K′ is less than 0.4, the results are not as satisfactory as when the value lies between 0.4 and 0.8. The second useful criterion concerns the nitrogen balance, which should differ significantly from the value obtained with the complete amino acid mixture, especially at full-N intake. Finally, the computed total nitrogen intake required for equilibrium (NI_e in table 1) should be significantly greater when one of the essential amino acids is critically reduced. As judged by these criteria, the phenylalanine experiments recorded in table 1 were carried out with a limiting concentration of this essential amino acid.

The response to feeding the complete amino acid mixture confirms many previous experiments in this laboratory. A conspicuous feature was the absence of any change in either fecal or urinary N excretion when the intake of N was dou-

TABLE 1

Average data for rats receiving amino acid diets

EXPERIMENT NUMBER	SERIES 260	SERIES 300				
	VI	I	II	III	IV	V
Amino acid mixture	1/8 L-phenylalanine	Complete	1/8 DL-phenylalanine	1/8 D-phenylalanine	1/8 L-phenylalanine	Complete
Number of rats	13	16	9	12	7	12
Average body weight (kg)	0.268	0.262	0.272	0.278	0.276	0.278
Metabolic body size ($kg^{¾}$)	0.372	0.366	0.377	0.383	0.381	0.382
	mg N/day/$kg^{¾}$					
Half-N period:						
N intake	69	72	71	68	70	69
Fecal N	29	40	31	30	28	29
Urinary N	117	110	124	126	120	108
N balance	− 76	− 78	− 84	− 88	− 79	− 68
Full-N period:						
N intake	130	143	137	133	135	132
Fecal N	28	37	28	29	26	25
Urinary N	134	114	143	148	146	103
N balance	− 33	− 8	− 36	− 43	− 37	+ 4
K′ (Half-N; full-N)[1]	0.72 ± 0.04	1.00 ± 0.04	0.75 ± 0.05	0.67 ± 0.05	0.64 ± 0.03	1.15 ± 0.03
NI_e[2]	178 ± 6	151 ± 4	187 ± 7	205 ± 10	193 ± 3	129 ± 2

[1] K′ is the slope of the line joining the half-N and full-N points when nitrogen balance is plotted against nitrogen intake.
[2] NI_e is the nitrogen intake computed to be necessary for attainment of nitrogen equilibrium.

bled. This fact indicates complete utilization of the increment in N intake and speaks well for the adequacy of the complete amino acid mixture for the maintenance of N equilibrium under these conditions. Periods I and V (series 300, table 1) were 20 weeks apart and the results illustrate the typical gradual improvement in N balance which occurs on a constant but low intake of the complete amino acid mixture. The full-N intake is fixed initially so that the average animal, even on the complete amino acid mixture, will not quite be able to attain N equilibrium in the early experiments of a series. At the end of 5 or 6 periods it often happens that N equilibrium, or a slight positive N balance, is attained, as is illustrated in experiment V, series 300. This phenomenon is also illustrated in earlier work from this laboratory (Nasset and Anderson, '50; Nasset et al., '51). The animal gives the most reliable results when near or well below the point of N equilibrium. For the reader who is unaccustomed to the unit of metabolic body size ($kg^{3/4}$), it may be helpful to state that the total N intake of the average 250-gm rat on a full-N diet was approximately 50 mg/day.

DISCUSSION

It is customary uniformly to apply statistical methods to the data and Fisher's "t" test has been applied to all comparisons of mean results. In comparing periods I and V of the series 300 rats, it is found that the K prime values for the two periods are not significantly different ($P > 0.2$). This means, of course, that the utilization of the increment in nitrogen intake remained unchanged over the 20-week period. The computation of the nitrogen required for maintenance of nitrogen equilibrium, however, is decidedly different. Table 1 indicates 151 mg nitrogen per day per $kg^{3/4}$ as the amount required for nitrogen equilibrium in period I. The same rats in period V required 129 mg per day per $kg^{3/4}$. This difference is highly significant ($P = 0.0005$). There can be no question about the improvement in nitrogen balance over this period.

The results in table 1 reveal that D-phenylalanine is rather well utilized for the maintenance of N balance in the adult rat. The slightly better responses obtained with DL- and L-phenylalanine were not statistically significant. It is logical to suppose, however, that no D-isomer of an amino acid should be quite as efficiently utilized as the corresponding L-isomer. The biological inversion of the unnatural compound involves certain hazards which could lead to losses along the way before the compound is ready to be incorporated into a protein molecule. It seems not unlikely that some of the keto acid resulting from oxidative deamination of the D-isomer, for example, might be lost through further oxidation before reamination and the subsequent synthetic processes were completed. The present experiments suggest but do not prove this possibility. On face value the results in table 1 show that the adult male rat under these conditions cannot differentiate between the two isomers of phenylalanine. According to Rose and Womack ('46) this is equally true of the growing rat.

It is interesting to note how closely similar the responses of two different lots of animals can be to identical experimental conditions. Experiment VI, series 260, and experiment IV, series 300, were both done with one-eighth L-phenylalanine amino acid mixtures, and the values for N balance, K′ and NI_e (table 1) for the two groups of rats agree remarkably well. Doubtless the tube feeding technique, which obviates the variables of appetite and spontaneous feeding, contributes greatly to the uniformity of results obtained in these experiments.

In previous work with valine, methionine and threonine (Nasset and Anderson, '50; Nasset et al., '51) it was demonstrated that nitrogen balance is a linear function of the intake of the limiting essential amino acid. This fact was used to compute the daily requirement of these amino acids for the maintenance of N equilibrium. In figure 1 N balance is plotted against phenylalanine N intake. It is evident that the two sets of points are closely grouped. An extrapola-

tion upward of the line joining the two average points obtained from one experiment yields a graphic estimate of the phenylalanine N required for maintenance of N equilibrium. A more satisfactory estimate is obtained by extrapolating the lines joining the individual points obtained for each rat for each experiment. The mean values thus obtained, expressed as milligrams phenylalanine N/day/kg$^{3/4}$, are: series 260, L- = 1.44 ± 0.05; series 300, DL- = 1.48 ± 0.06, D- = 1.63 ± 0.08, and L- = 1.54 ± 0.02. The weighted mean for the two

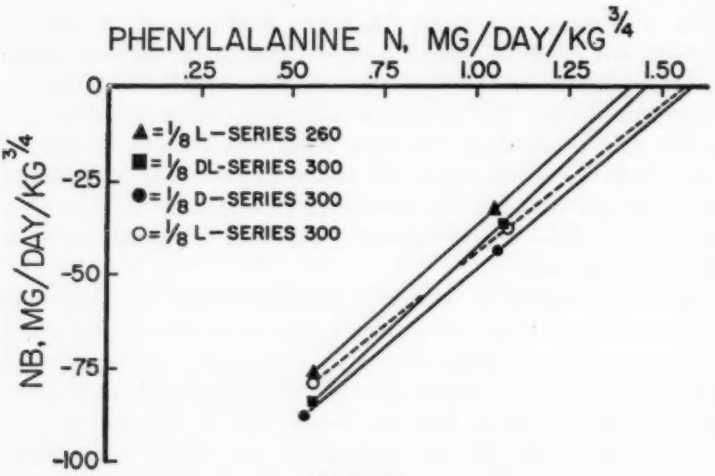

Figure 1

experiments with L-phenylalanine is precisely the same as the DL- value. In terms of the amino acid, therefore, the requirements for the maintenance of N equilibrium under these conditions are: 17.5 mg of L- or DL-phenylalanine/day/kg$^{3/4}$ and 19.2 mg of D-phenylalanine/day/kg$^{3/4}$. The indicated 10% greater requirement for the unnatural isomer is suggestive but statistically not significantly different from the value for the natural and racemic forms ($P = 0.15$).

Benditt et al. ('50) state that the adult male albino rat (Sprague-Dawley) requires 3.1 mg phenylalanine/day/100 cm^2

of body surface for the maintenance of N equilibrium. Unfortunately they failed to record the body weights or the actual food consumption of the three rats which were used for this determination. A direct comparison with the results of the present investigation is not very precise but probably still worthwhile. Assuming that the average body weight of the rats used by Benditt et al. was 318 gm (mean of the range given; i.e., 283 to 353 gm) the surface area, according to Lee's formula ('29), which they used, is 397.5 cm^2. According to their results, therefore, the daily requirement for the average rat is 12.3 mg of DL-phenylalanine per day. The metabolic body size of this average rat is 0.423 $kg^{3/4}$ and the requirement is 29.1 mg of DL-phenylalanine/day/$kg^{3/4}$, which is 66% greater than the requirement computed in the present investigation.

Rose ('49) tentatively gave 1.10 gm of L-phenylalanine as the minimum daily requirement for the adult human male. Assuming the average body weight of his subjects to be 70 kg, the requirement is 45.5 mg of L-phenylalanine/day/$kg^{3/4}$. This computation suggests that the human requirement is 160% greater than that for the rat as established in the present investigation. This may represent a real species difference but it is impossible to decide this point until experimental details of the human experiments become available. One reason for the high value given by Rose is the fact that what he reports as a minimum value is not a mean of his minima but actually his highest minimum among the group of subjects who ingested any particular amino acid. If the mean of the minimum values and the body weights of the subjects were available, it would be possible to make more valid interspecific comparisons.

SUMMARY

Nitrogen balance was determined on two groups of adult male albino rats which derived all of their dietary nitrogen from mixtures of amino acids. Each experiment included the

following dietary regimes in the order given: 14 days on maintenance diet (9.6% whole egg protein); 7 days on N-free diet; 7 days on amino acid diet supplying approximately half of the maintenance requirement of total nitrogen; 7 days on double the quantity of the amino acid mixture fed in the previous period. These diets, except the maintenance diet, were fed by stomach tube in two equal portions daily, and each rat received the same quantity of diet each day.

If the phenylalanine in a complete mixture of essential amino acids is reduced sufficiently, the nitrogen balance is adversely affected. When this amino acid is the limiting factor in the utilization of dietary nitrogen, it is assumed that N balance is a linear function of phenylalanine intake. On this assumption, the requirement for maintenance of N equilibrium is 17.5 mg of L- or DL- and 19.2 mg of D-phenylalanine/day/$kg^{3/4}$.

Other values for the adult male rat and for the adult human male, computed from the data of Benditt et al. ('50) and Rose ('49), are 29.1 mg/day/$kg^{3/4}$ and 45.5 mg/day/$kg^{3/4}$ for DL- and L-phenylalanine, respectively.

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APPLICATION OF THE CHROMIC OXIDE INDICATOR METHOD TO BALANCE STUDIES WITH GROWING CHICKENS

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TWO FIGURES

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The determination of a nutrient balance is a basic technique which has been applied extensively in nutritional studies of digestibility, utilization and retention. The procedure most frequently followed is to collect all the excrement produced during the experimental period and, by comparison with the total food intake, to determine the "disappearance" of the nutrient under study. This procedure is laborious and time-consuming.

The basic data needed to establish a balance comprise the amounts of excrement derived from the measured food intake over a period of time sufficiently long to give a representative sample characteristic of the experimental conditions employed. The procedure can be facilitated by any method which will aid in establishing the amount of excrement equivalent to a given amount of food. In one such method, a known amount of a completely indigestible index material is incorporated in the diet; the amount of food from which a sample of feces was derived can be calculated from the respective concentrations of index material in food and feces. Chromic oxide (Edin, '18; Edin et al., '44; Schürch et al., '50), iron oxide (Bergeim, '26), barium sulfate (Whitson et al., '43) and lignin (Kane et al., '50) have been employed as index substances.

Less work in this field has been done with poultry than with other species. Determination of true digestibility with birds is complicated by the fact that urinary and fecal excrements are voided together.

The studies which have been made are typified by those of Whitson et al. ('43) and Olsson ('50). The former workers used barium sulfate as the index material in determining fat utilization by growing chickens. Olsson applied the "Edin Indicator Method" to digestibility studies with mature hens, geese, and turkeys on restricted dietary regimes. In both cases the balance experiments were carried out on birds kept in individual cages.

The insoluble nature of chromic oxide and the practicability of its use as an index substance have been well established in the studies already referred to. The purpose of the present work was to define the conditions of its applicability to balance studies with groups of growing chicks under ad libitum feeding conditions, with particular reference to the following: (1) Length of collection period necessary to assure a representative sample of excrement; (2) reproducibility of results in successive collection periods; (3) recovery of ingested chromic oxide, and (4) comparison of balance data by the index and total collection methods.

In the course of these studies the colorimetric method of Schürch and co-workers ('50) for determining chromium was modified to improve its sensitivity.

EXPERIMENTAL PROCEDURE AND RESULTS

Analytical method

Colorimetric analysis of chromium, first developed by Paloheimo and Paloheimo ('35) and modified by Schürch et al. ('50), has the advantages over other methods of speed and simplicity, so desirable in a routine method to be applied to a large number of samples. The method used in this laboratory is essentially that of Schürch and co-workers except for the measurement of color density. The sample of diet or excre-

ment is ashed, and the ash fused with sodium peroxide to convert chromic oxide to chromate. After appropriate dilution, the color density is measured in a Beckman DU spectrophotometer. Determination of the absorption spectrum of

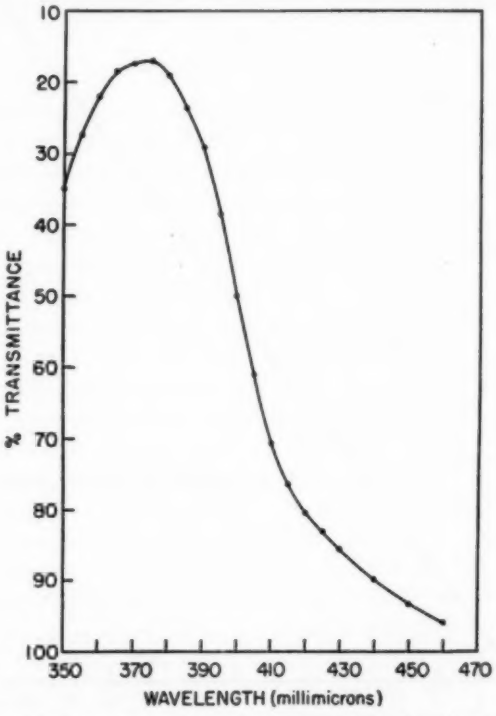

Fig. 1 Absorption spectrum of chromate from fused chromic oxide (concentration equivalent to 12 μg chromic oxide per milliliter).

such a solution prepared from fused chromic oxide showed a single absorption maximum at 375 mμ (fig. 1). A standard reference curve was established at this wavelength by determining the optical density of solutions prepared from known amounts of chromic oxide carried through the fusion process. Figure 2 shows the linear relation between the optical density

of the chromate solution and the concentration of chromium, expressed as the amount of chromic oxide prior to fusion; the linear regression was derived statistically from data based on three or more replicate determinations at each of 7

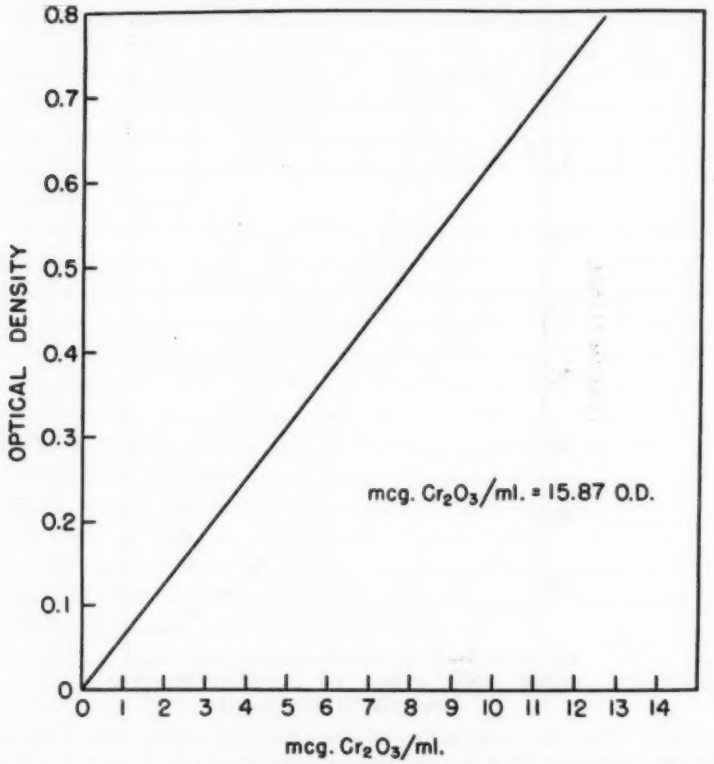

Fig. 2 Linear relation at 375 mμ between optical density and chromium content of solutions derived from fused chromic oxide.

chromium concentrations. In contrast to the results reported by Schürch and co-workers, determining the color density at 375 mμ gives excellent conformity with the Lambert-Beer law, and also increases the sensitivity of the method materially. The standard deviation of a single determination, derived

from the data used to establish the reference curve, was found to be $\pm$ 0.11 µg chromic oxide per milliliter for concentrations of up to 12 µg per milliliter. Precision tended to decrease above this concentration, as indicated by a disproportionate increase in standard deviation, possibly due to deviation from the Lambert-Beer law.

Feeding experiments

Chromic oxide was incorporated in the experimental diets either as the pure powder at a 1% level (experiment I), or as a chromic oxide-flour-water bread prepared as suggested by Kane and co-workers ('50) and finely ground in a Wiley mill. The latter procedure was employed in experiments II and III, using a level of chromic oxide bread sufficient to supply 0.2% chromic oxide in the diet.

The ration used in experiment I was composed of natural feedstuffs and was typical of rations commonly used for young chicks under farm conditions. A more highly digestible semi-purified ration was used in experiments II and III. It contained cerelose and casein in addition to ground corn and wheat, soybean flour, fish meal, yeast, and mineral and vitamin supplements. Except for the higher digestibility of the latter ration, which will be evident in the data presented, the results were not influenced by the nature of the rations.

Groups of 10 to 20 chicks between 4 and 5 weeks of age were used in these studies, housed in cages with wire floors 3.5 by 4.5 feet in size. The diets containing chromic oxide were fed for a three-day preliminary period, after which time collections of excrement were made, using galvanized sheet metal trays suspended beneath the cages. Daily measurements of feed consumption were made and particular care was taken to account for all feed wastage by attaching shallow screened trays beneath the feeders to catch feed spilled by the chicks. This procedure also effectively minimized contamination of excrement with feed.

Collections of excrement were made as quantitatively as the conditions permitted at intervals of 8 to 24 hours, with

arrangement of periods such that the data could be grouped into successive 24-hour periods. The excreta were frozen immediately after collection and dried in the frozen state in a Dessivac apparatus.

In theory, a small sample of excrement would be sufficient to provide the data for calculating the retention or disappearance of a nutrient. The physiological processes of the chicken do not result in the production of excrement of uniform composition throughout a 24-hour period, however, primarily because of diurnal rhythm in the elimination of cecal droppings which contain only traces of urinary excreta (Herrick and Edgar, '47). Collections during periods of less than 16

TABLE 1

Chromic oxide content of excrement in successive 24-hour periods

COLLECTION PERIOD	CHROMIC OXIDE CONTENT Experiment		
	I	II	III
	%	%	%
1	2.70	1.04	1.11
2	2.60	1.03	1.03
3	2.56	0.84	1.00
4		1.05	1.17

hours showed excessive variability of chromium content; to conserve space, these data are not reported in detail. Instead, the data have been combined into 24-hour periods.

Table 1 shows the chromic oxide content of the excreta on a moisture-free basis for successive 24-hour periods in the three experiments. The precision of the colorimetric determination of chromium is such that practically all of the variations from period to period can be considered to be due to variability among the animals and to feeding conditions. In the main the data show excellent agreement in successive collection periods, indicating that 24-hour periods are satisfactory units for this type of work.

The two exceptions, 0.84% in experiment II and 1.17% in experiment III, are probably due to changes in level of feed

intake, as shown in table 2. The value of 0.84% in experiment II was obtained during a 24-hour period of elevated feed intake and increased output of excreta. The reasons for the change in consumption were not evident, but since a temporarily higher plane of nutrition was produced it would be expected that digestive efficiency would be somewhat lower. Conversely, in experiment III the high value of 1.17% chromic oxide occurred in a period when feed intake was purposely reduced and production of excreta was low. This temporarily lowered plane of nutrition was accompanied by an increased

TABLE 2

The effect of feed intake on output of feces and fecal level of chromic oxide

COLLECTION PERIOD	FEED INTAKE	FECES OUTPUT	Cr_2O_3 IN FECES
	gm	gm	%
	Experiment II		
1	1,056	244	1.04
2	1,137	243	1.03
3	1,191	288	0.84
4	1,155	258	1.05
	Experiment III		
1	1,067	240	1.11
2	1,140	240	1.03
3	1,158	252	1.00
4	941	148	1.17

efficiency of utilization of the diet. These exceptions to the general uniformity of fecal levels of chromic oxide show that a single collection period may not produce a representative excrement sample. Agreement in the chromic oxide content of two or more successive 24-hour collections of excrement can be used as a criterion of the uniformity of conditions and the validity of the balance measurement.

Data are presented in table 3 showing the recovery of ingested chromic oxide in the feces. Large fluctuations between periods were observed, but over the entire three or 4 days of each experiment approximately 95% of the ingested chromic

oxide was recovered in the excrement. Recovery for experiment III was 96% when the low value for period 4 was omitted from the calculations; omission of this period is probably justified for the reasons discussed above. Incomplete recovery of ingested chromic oxide could have been due to (1) incomplete collection of feces, caused in part by the large size of the cages used, and (2) the somewhat larger capacity of the digestive tract of the rapidly growing birds at the end of the period as compared to the beginning. Recovery of 99% of the chromic oxide ingested by hens maintained in special individual cages has been reported by Olsson ('50). Failure

TABLE 3

Recovery of ingested chromic oxide in successive 24-hour periods

COLLECTION PERIOD	RECOVERY OF CHROMIC OXIDE Experiment		
	I	II	III
	%	%	%
1	91	102	105
2	127	94	91
3	76	87	91
4		100	77
Total recovery	94	95	92

to obtain this degree of recovery in the present work was probably due mainly to the difficulty of achieving quantitative collection of excrement under the conditions employed. More striking than the extent of recovery over the entire period were the extreme daily variations, due probably to the fact that fecal output during a given 24-hour period does not represent the feed consumed in the same period. These data demonstrate a major advantage of the index method over the method of total collection in studies covering relatively short time intervals.

The results of applying the chromic oxide data to calculation of dry matter utilization and nitrogen retention are

shown in tables 4 and 5 in comparison with the balances determined by measurement of the total intake and excretion. With the exceptions noted above, the successive 24-hour collections showed uniform utilization values. Comparison of the average values determined by the index method with the values determined by measuring total intake and excretion shows a higher degree of utilization found by the latter method in every case. This is probably due to the fact that any failure to achieve quantitative collection of excrement would result in the finding of a higher apparent degree of utilization by the

TABLE 4

Dry matter utilization determined by index method in comparison to total measurement of intake and excretion

COLLECTION PERIOD	UTILIZATION OF DRY MATTER BY INDEX METHOD Experiment		
	I	II	III
	%	%	%
1	61.9	77.5	78.6
2	60.4	77.3	76.9
3	59.8	72.1	76.2
4		77.7	79.7
Average	60.7	76.2	77.8
Calculated from total measurement	63.1	77.3	79.6

total collection method, but would not affect the results obtained by the index method. The discrepancy is of the same order of magnitude as the amount of ingested chromic oxide unaccounted for in the total balance (table 3). As would be expected from the data in table 3, calculation of the utilization of dry matter and nitrogen for 24-hour periods by the total collection method gave highly variable results for successive periods, in contrast to the relatively uniform results obtained by the index method.

Nitrogen retention values calculated by the index method were lower than those determined by the total collection method to an extent greater than the apparent loss of in-

gested chromic oxide. This suggests that the excreta lost by adherence to the screen floors was relatively richer in chromic oxide than nitrogen, with the result that the nitrogen excretion per unit of chromic oxide was erroneously high and the nitrogen retention correspondingly low. This could occur if cecal droppings were preferentially lost. Their physical nature is such that they would tend to adhere to the screen floors more readily than normal droppings. This explanation is

TABLE 5

Nitrogen retention determined by index method in comparison to total measurement of intake and excretion

COLLECTION PERIOD	NITROGEN RETAINED BY INDEX METHOD Experiment	
	II	III
	%	%
1	48.02	47.26
2	47.29	40.61
3	37.53	42.88
4	45.84	50.17
Average	44.67	45.23
Calculated from total measurement	47.26	49.66

consistent with the observation that the dry matter balance by the two methods of determination disagreed less than would be expected by the extent of loss of the ingested chromic oxide.

SUMMARY

The colorimetric method of Schürch and co-workers for the determination of chromium has been modified by measurement of color density at 375 mμ. With this modification, the method is highly sensitive and conforms to the Lambert-Beer law.

The index method of conducting measurements of nutrient balance using chromic oxide has been applied to groups of growing chickens under ad libitum feeding conditions.

Collecting excrement in unit periods of 24 hours is satisfactory for circumventing the effect of the diurnal rhythm of excretion of cecal droppings. Changes in level of feed intake are reflected quickly in the level of chromic oxide in the excrement, with the result that any single 24-hour collection may be subject to serious error. A useful criterion for determining the validity of a balance obtained by this method is the agreement between two or more successive 24-hour periods in the chromic oxide content of the excrement.

Compared to results obtained by measurement of total intake and excretion, the index method gave more consistent and probably more accurate results of balance measurements under the conditions employed.

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VITAMIN B_{12} REQUIREMENT OF WEANLING PIGS

II. PERFORMANCE ON LOW LEVELS OF VITAMIN B_{12} AND REQUIREMENT FOR OPTIMUM GROWTH [1, 2]

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In a previous study, Richardson et al. ('51) found that the addition of 5 μg of crystalline vitamin B_{12} per pound of an otherwise well-fortified corn-soybean oil meal ration in the presence of a combination of antibiotics produced just as efficient and rapid gains with growing-fattening pigs as did the addition of 10 or 20 μg. The authors found that there are sufficient storages of vitamin B_{12} in weanling pigs, based upon weight gains, to produce effective nutritional "carry-over" for three to 8 weeks.

The present study was conducted to investigate the performance of weanling pigs on low levels of vitamin B_{12} and establish further the pig's requirement of vitamin B_{12} for optimum growth.

EXPERIMENTAL PROCEDURE

The same 18% plant protein basal ration was used in this experiment as in the previous one (Richardson et al., '51) except that 33.0 mg ascorbic acid were added per pound of

[1] The crystalline vitamin B_{12}, streptomycin sulfate and procaine penicillin G and all vitamins, except vitamins A and D_3 and folic acid, were supplied by Merck and Co., Inc., Rahway, New Jersey. The vitamin D_3 was supplied by Standard Brands, Inc., New York, New York; the aureomycin hydrochloride and folic acid by Lederle Laboratories, Pearl River, New York; and the terramycin hydrochloride by Chas. Pfizer and Co., Inc., Brooklyn, New York. The assistance of the Meat Laboratory in obtaining the tissue samples is acknowledged.

[2] Journal Paper J-2061 of the Iowa Agricultural Experiment Station, Ames. Project 930.

ration in this experiment. The ration was composed of ground yellow corn, solvent soybean oil meal (blended), essential minerals, trace minerals, vitamins A and D_2 and 13 water-soluble vitamins. To this ration were added 0, 2, 4 and 6 μg of crystalline vitamin B_{12} per pound of total ration for different individually fed pigs. An antibiotic mixture, containing 20 mg (twice the amount used in the first study) each of aureomycin hydrochloride, terramycin hydrochloride, streptomycin sulfate and procaine penicillin G per pound of total ration, was included in the ration fed to all the pigs.

Six sets of 4 litter-mate pigs were randomly allotted to the 4 ration treatments. Five of the litters were Duroc and the 6th (litter 710) was a Landrace × Duroc cross. As is indicated in table 1, three of the litters were barrows and three were gilts. The pigs were weaned at 56 days and placed on the basal ration for 6 days before starting on their respective experimental rations. During this period fecal, urine and blood samples were collected and the pigs were wormed with sodium fluoride and sprayed with benzene hexachloride. The 6 pigs on the zero level of vitamin B_{12} averaged 23.0 lb., those on the 2 μg level 23.3 lb., those on the 4 μg level 25.5 lb., and those on the 6 μg level 24.7 lb. at the start of the experiment. The pigs were confined in 18-by-40-inch wire-floored, wooden crates to 100 lb. live weight. Feed and water were offered ad libitum.

The dams of these pigs had been fed an adequate 16% protein ration containing sources of animal protein, minerals, trace minerals and vitamins A and D_2, riboflavin, niacin and pantothenic acid during gestation. During lactation they received a plant protein ration which was fortified only with vitamins A and D_2 and minerals, in order that the pigs would be semi-depleted of water-soluble vitamins (including vitamin B_{12}) when started on experiment.

Blood, urine and fecal samples were collected from each pig at 100 lb. live weight or when removed from experiment. Three pigs fed each level of vitamin B_{12} were slaughtered after reaching 100 lb. live weight to obtain the liver and intestinal tract contents for vitamin B_{12} assay.

The vitamin B_{12} assays were made by using a modified basal medium of Peeler et al. ('49) and the procedure of Friedland ('51). *Lactobacillus leichmannii* 313 was used as the assay organism. The results of these assays are reported as vitamin B_{12}; however, the reader should bear in mind that it might be more correct to refer to these values in terms of response to the *Lactobacillus leichmannii* organisms. Cer-

TABLE 1

Summary of gains and feed data to 75 and 100 lb. live weight

LEVEL OF VITAMIN B_{12} (μg per lb. ration)	INITIAL TO 75 LB. LIVE WT.				INITIAL TO 100 LB. LIVE WT.			
	0	2	4	6	0	2	4	6
Individual pigs								
Ave. daily gains (lb.)								
Litter 710 – B [1]	1.11	1.18	1.33	1.74	0.83	0.92	1.41	1.55
Litter 730 – G	0.56	1.10	1.26	1.32	... [2]	1.11	1.38	1.41
Litter 770 – G	1.02	1.09	1.33	1.17	0.98	1.15	1.40	1.21
Litter 900 – B	0.93	1.04	1.46	... [3]	... [2]	1.10	1.40	... [3]
Litter 950 – B	1.30	1.54	1.50	1.53	1.04	1.51	.49	1.36
Litter 960 – G	0.93	1.36	1.18	1.31	... [2]	1.40	1.15	1.32
Treatment averages								
Ave. daily gains (lb.)	0.98	1.22	1.34	1.41	...	1.20	1.37	1.37
Ave. daily feed (lb.)	2.90	3.28	3.35	3.40	...	3.62	3.85	3.97
Feed per 100 lb. gain (lb.)	309	270	249	241	...	303	281	277

[1] B — barrow and G — gilt.

[2] These pigs developed growth stasis, were removed from experiment, and were then injected with or fed vitamin B_{12}.

[3] This pig did not react normally to the experimental treatment, lost weight and died after 8 days on experiment.

tain techniques were employed, namely paper strip chromatography, alkaline hydrolysis, enzymatic digestion and dialysis, to verify that vitamin B_{12} was the active principle being studied.

RESULTS AND DISCUSSION

The average individual daily gains of the pigs to 75 and 100 lb. live weight are presented in table 1. An analysis of variance of the average daily gains to 75 lb. revealed that the

differences among rations, as measured by the linear regression on level of vitamin B_{12}, were significant at the 1% probability level. The average daily gain of 1.74 lb. for the pig in litter 710 receiving 6 μg of vitamin B_{12} per pound of ration is unusually high for a pig of this age and body weight. If the gain of this pig had been consistent with the daily gains of other pigs receiving 6 μg of vitamin B_{12} per pound of ration, the average daily gains to 75 lb. would be the same for the pigs receiving 4 and 6 μg of vitamin B_{12} per pound of ration.

The average daily gains of the pigs to 100 lb. live weight were 1.37 lb. for the pigs receiving 4 and 6 μg of vitamin B_{12} per pound of ration. These pigs appeared normal in every way and did not show any vitamin B_{12} deficiency symptoms. The pigs receiving 2 μg of vitamin B_{12} per pound of ration made an average daily gain of 1.20 lb. to 100 lb. live weight. These pigs did not have the "bloom" or thrifty general appearance of those receiving 4 and 6 μg of vitamin B_{12}, but none of them developed clear-cut vitamin B_{12} deficiency symptoms.

The pigs fed the basal ration developed a rough skin and haircoat and were generally unthrifty in appearance. Three of the basal-fed pigs required an average of 34.3 days to reach 100 lb. live weight from a weight of 75 lb. The other three developed growth stasis and were very emaciated in appearance. One pig had started to lose weight. Two of the pigs fed the basal ration were injected intraperitoneally with 15 μg of vitamin B_{12} and 6 μg were added per pound of their ration. During the next 17 days these two pigs made gains of 20 and 27 lb. on 58 lb. of feed per pig. These are average daily gains of 1.18 and 1.59 lb., as compared with 0.76 and 0.78 lb., respectively, before receiving vitamin B_{12}. Six micrograms of vitamin B_{12} were added to each pound of ration fed the other vitamin B_{12}-deficient pig. It gained 17 lb. in 12 days on 50 lb. of feed. This is an average daily gain of 1.42 lb., as compared with 0.56 lb. before receiving vitamin B_{12}. In this case, feeding of vitamin B_{12} appeared to be just as effective for recovery as injection. These results

indicate that vitamin B_{12} must be supplied in the ration of the growing pig if it is to attain optimum growth.

The average daily feed consumed per pig and the feed required per 100 lb. gain are given in table 1. In general, the pigs which gained the fastest ate the most feed. The correlation between average daily gains and average daily feed consumed per pig to 75 lb. live weight ($r = 0.8187$) was significant at $P = 0.01$. However, there were no significant differences between the daily feed consumption of the pigs on the various vitamin B_{12} levels. There was an increase in feed efficiency with each addition of vitamin B_{12} to the ration, significant at $P = 0.05$, measured by the linear regression.

Summaries of the blood data and results of the vitamin B_{12} assays of the liver, urine, feces and digestive tract contents are given in table 2.

The addition of vitamin B_{12} to the basal ration did not produce significant changes in hemoglobin values, which is in agreement with the findings of Beeson and Bloss ('49) and Bowland et al. ('50). There were no significant differences in the red and white blood cell counts. All were within the normal range. The total nitrogen was lowest and urea highest in the blood of pigs fed the basal ration. There was a decrease in blood urea as the amount of vitamin B_{12} fed was increased. Apparently vitamin B_{12} enhanced the absorption of amino acids or nitrogen as well as aiding in the utilization of amino acids by the tissues. These results are in agreement with the work of Abbott ('51) with rabbits and Charkey et al. ('50) and Stern and McGinnis ('51) with chicks, in which they found that vitamin B_{12} enhances the utilization of nitrogen.

Twelve pigs, three from each level of vitamin B_{12}, were slaughtered after reaching 100 lb. live weight. The average weights of the livers decreased as the vitamin B_{12} in the feed was increased, but the amount of vitamin B_{12} per gram of liver increased. The average total amount of vitamin B_{12} per liver from pigs fed 4 and 6 μg of vitamin B_{12} per pound

of ration was practically double that found in the livers of pigs fed 0 and 2 μg.

The amount of vitamin B_{12} per gram of feces showed very little variation among treatments. The total amount of vitamin B_{12} varied in proportion to the total feces voided. The most intriguing part of the urinary and fecal assays is that they consistently showed greater vitamin B_{12} values than could possibly be accounted for by vitamin B_{12} in the ration. This definitely points to synthesis of vitamin B_{12} in the digestive tract of the pig. A pilot experiment, summarized in table 2, in which ingesta from the small intestine, cecum and large intestine of 12 pigs were assayed, indicated that vitamin B_{12}, or a vitamin B_{12}-like substance or substances, is synthesized in the lower part of the digestive tract of the pig even in the presence of high levels of antibiotics.

SUMMARY AND CONCLUSIONS

Six sets of 4 litter-mate pigs, three barrows and three gilts, were individually fed and watered ad libitum from weaning to 100 lb. live weight, confined in wire-floored wooden crates. The pigs were fed a fortified corn-soybean oil meal ration containing 80 mg of a combination of antibiotics per pound of ration. To this ration were added 0, 2, 4, and 6 μg of crystalline vitamin B_{12} per pound.

The addition of vitamin B_{12} to the basal ration produced significantly greater gains to either 75 or 100 lb. live weight. There was practically no difference in the average daily gains of the pigs on the 4- and 6-μg levels of vitamin B_{12}. Vitamin B_{12}-deficient pigs on the basal ration exhibited a dramatic response to the intraperitoneal injection or oral feeding of crystalline vitamin B_{12}, or both. Vitamin B_{12} also significantly increased feed efficiency.

There were no significant differences in hemoglobin or red and white blood cell counts of pigs fed different levels of vitamin B_{12}. The pigs fed the basal ration had the lowest level of total nitrogen and the highest level of urea in the blood. The amount of blood urea decreased as the amount

of vitamin B_{12} in the ration was increased. There was a tendency for the livers of the pigs to become lighter in weight as the amount of vitamin B_{12} fed was increased; however, the amount of vitamin B_{12} per gram of liver and total vitamin B_{12} per liver increased. Urinary vitamin B_{12} assay val-

TABLE 2

Summary of blood data and vitamin B_{12} content of liver, urine, feces and digestive tract contents

LEVELS OF VITAMIN B_{12} (μg per lb. ration)	0	2	4	6
	Blood [1]			
Hemoglobin (gm per 100 ml):				
Initial	6.1	5.8	6.3	6.1
Final	13.3	13.6	13.3	13.2
Blood cell counts:				
Red (× 1000)	7,127	6,093	7,388	7,018
White	15,540	21,037	18,360	24,728
Total nitrogen (gm per 100 ml)	3.08	3.23	3.23	3.26
Urea (mg %)	13.5	10.8	10.4	9.5
	Liver [2]			
Total weight (gm)	1,154	1,050	1,013	842
B_{12} per gm (mμg)	49	63	110	135
Total B_{12} per liver (μg)	57	66	111	114
	Urine [2]			
Total in 24 hours (ml)	1,667	2,722	2,670	2,766
B_{12} per ml (mμg)	0.55	1.90	1.05	1.05
Total B_{12} in 24 hours (mμg)	917	5,172	2,804	2,904
	Feces [2]			
Total in 24 hours (gm)	459	973	1,487	1,045
B_{12} per gm (μg)	0.92	0.85	0.85	0.95
Total B_{12} in 24 hours (μg)	422	827	1,264	993
	Digestive tract contents [2]			
B_{12} per gm (mμg):				
Small intestine	12	24	28	34
Cecum	407	237	287	293
Large intestine	893	833	388	783

[1] Averages of 6 pigs on the 0-, 2- and 4-μg levels and 5 pigs on the 6-μg level of vitamin B_{12}.

[2] Averages of three pigs per treatment.

ues were inconsistent, thereby indicating a difference among pigs in their ability to synthesize this nutrient. Differences in the amount of vitamin B_{12} per gram of feces were small. The total amount varied with the total feces voided. Assay of intestinal contents from 12 pigs showed synthesis of vitamin B_{12} or vitamin B_{12}-like substances in the lower part of the digestive tract of the pig.

These results indicate that the vitamin B_{12} requirement of the weanling pig for efficient weight gain is at least 4 μg per pound of total ration when added to a corn-soybean oil meal ration containing a combination of antibiotics for partial control of intestinal microflora.

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